Predictive model development and lag phase characterisation for applications in the meat industry

by

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DECLARATION

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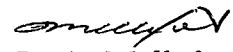

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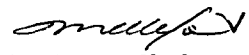

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ABSTRACT

Foods of bovine origin have been implicated as the principle vehicle in disease outbreaks of pathogenic *Escherichia coli*, hence the increasing interest in understanding the behaviour of this pathogen on carcasses during processing and handling. In the absence of rapid and non-invasive methods to determine the microbiological safety of meat carcasses, predictive modelling has been suggested as a strategy to estimate the consequences of processing and handling procedures on the fate and numbers of microorganisms. This thesis reports two contributions in realising the potential of predictive modelling for use in the food industry: development and extensive validation of a growth model for *E. coli* and advances in characterisation of bacterial lag phase duration.

A modified square-root model for predicting *E. coli* growth was developed and its reliability assessed against a variety of data. The bias and accuracy factors formed the basis of the model evaluations and provided an objective summary of model performance. The model performed well when compared to data generated in liquid growth media, ground beef and for data collated from the literature. The model performed as well as, or better than other published models for *E. coli* growth. In particular, the model predicted growth in meat and meat products better than other models.

Evaluation of predictive models in industry is considered the most rigorous test of model performance. The deliberate introduction of potential pathogens in abattoirs is precluded in Australia, thus the *E. coli* growth model could not be validated by trials on carcasses during normal commercial processing. Instead, a predictive growth model for a surrogate organism, *Klebsiella oxytoca*, was developed and evaluated against data for the growth of *K. oxytoca* on carcasses during normal chilling operations. Those studies suggest that predictive modelling can be used to predict the *average* changes in numbers of bacteria on a carcass

resulting from temperature and water activity changes caused by air chilling processes.

Lag times have long been considered an uncontrollable variable in food microbiology. Studies were undertaken to describe the effects of environment and physiological history of the cell on lag times. Abrupt temperature, pH and osmotic shifts of cultures were found to induce lag phases in a variety of foodborne bacteria, highlighting the prospect of inducing lags by manipulating the rate and extent of change of environmental conditions. Variability in bacterial lag times was reduced by using the concept of relative lag times or "generation time equivalents", i.e. the ratio of lag time to generation time (RLT). The physiological history of the cell, including growth phase and habituation, affect the magnitude of the RLT response. In general, environmental downshifts induce larger RLTs than equivalent upshifts. These observations support the hypothesis that lag time can be understood in terms of the *amount* of work to be done to adjust to new environmental conditions and the *rate* at which that work is done. The results in this thesis demonstrate that careful interpretation of RLT responses under very stressful environmental conditions is required due to potential changes in growth curve shapes. Additionally a normal physiological range for water activity is proposed.

Characterisation of bacterial lag times using RLT simplifies their inclusion in growth predictions, thus increasing the utility of predictive models. Results in this thesis support those of Ross (1999) who observed a common pattern of distribution of relative lag times for a wide range of species across a wide range of conditions in the range of 4 to 6 generation time equivalents.

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CONTENTS

ABBREVIATIONS

%T	Per cent transmittance
A.	Appendix
Af	Accuracy factor
a_w	Water activity
Bf	Bias factor
BHA	Brain Heart Infusion Agar
BHAP	BHA with 0.1% pyruvate
BHI	Brain Heart Infusion Broth
BHI-85	BHI with 85mM lactic acid, pH5.55
BHI-85-S	BHI with 8% NaCl and 85mM lactic acid, pH5.55
BHI-130	BHI with 130mM lactic acid, pH6.1
BHI-130-S	BHI with 8% NaCl and 130mM lactic acid, pH6.1
CFU or cfu	Colony forming unit
CHBA	Columbia Horse Blood Agar
EMB	Eosin Methylene Blue Agar
EMB-S	EMB containing 100μg.mL^{-1} streptomycin
FMM	Food MicroModel
Est.	Estimate
Eqn.	Equation
GT	Generation time
MAC	MacConkey Agar
max	maximum
min	minimum
MM	Minimal medium
MPD	Maximum population density
NB	Nutrient Broth
NB-2S	NB containing 200μg.mL^{-1} streptomycin
NSG	Non-sustainable growth
OD	Optical density
PCA	Plate Count Agar
PCA-S	PCA with 100μg.ml^{-1} streptomycin
PCA-NaCl	PCA with 50g.L^{-1} NaCl
PECC	3M™Petrifilm™ *E. coli* Count plates
*p*FMM-*Ec*	A proxy polynomial model for Food MicroModel-*E. coli*
PMP	Pathogen Modeling Program
PS	Physiological saline (i.e. 0.85% NaCl)
PW	0.1% Peptone water
PWS	0.1% Peptone water with 5% NaCl
RLT(s)	Relative lag time(s)
RMSE	Root mean square error
S.	Section
TGI	Thermal gradient incubator
TSB-YE	Tryptone Soya Broth with 0.6% yeast extract
VC	Viable count
[LAC]	Lactic acid concentration
Δ%T	Change in percent transmittance
ΔOD	Change in optical density

1. INTRODUCTION

The provision of a safe and wholesome food supply is considered a basic tenet of a developed nation (McMeekin and Olley, 1995), yet the incidence of foodborne disease in the developed world is increasing and new pathogens continue to emerge (Maurice, 1994). The increased incidence of food poisoning has been attributed to many things, including: changes in agricultural practices; pressure for less additives in foods; changes in food preservation operations; proliferation of international trade in food products; increased population mobility and changes in consumer eating habits, with a demise in home cooking and corresponding growth in the mass catering sector (Maurice, 1994; McMeekin and Olley, 1995). The emergence of new foodborne pathogens is facilitated by pathogens mutating, changes in population demographics, with an increase in the number of immunocompromised members of the population, and the fact that reservoirs for pathogens are often major food sources.

Of the many pathogens that have emerged recently, a strain of *Escherichia coli* (*E. coli*), O157:H7, is especially virulent and has a well publicised relationship with foods of bovine origin. This has prompted renewed worldwide interest in pathogenic *E. coli* and meat hygiene. Currently available dressing procedures cannot be relied upon to prevent or remove all of the bacterial contamination on the carcass surface (Vanderlinde *et al.*, 1998). As the carcass surface is inevitably contaminated with bacteria, steps are taken to minimise proliferation, usually by rapid chilling. Temperature is known to be an important factor which influences the rate of growth of bacteria on carcass surfaces. Chilling techniques such as those employed in Australia, however, introduce a second constraint, water activity (a_w), due to surface drying of the carcass.

Despite the advent of rapid genetic and immunological techniques for detecting foodborne pathogens, assessment of the microbiological quality of foods is usually a retrospective process and is therefore only partially effective in protecting consumers from foodborne hazards. Predictive modelling was developed as an adjunct to traditional microbiological techniques. The survival and/or growth of an organism of concern may be predicted on the basis of a mathematical relationship between

microbial growth rate and environmental conditions (McMeekin *et al.*, 1993). The successful application of predictive modelling is entirely dependent on the development and validation of appropriate models (McMeekin and Olley, 1995).

This thesis investigates two significant aspects of predictive modelling; model validation and characterisation of bacterial lag times.

1.1 ESCHERICHIA COLI

E. coli is a member of the family Enterobacteriaceae and constitutes a part of the normal, facultatively anaerobic microbiota of the intestinal tracts of humans and warm-blooded animals (Doyle *et al.*, 1997). *E. coli* isolates may be differentiated serologically on the basis of 3 major surface antigens; the O (somatic), H (flagellar) and K (capsular) antigens (Doyle *et al.*, 1997). Some strains of *E. coli* are pathogenic and cause diarrhoeal illness in humans. Currently, six serologically distinct groups of *E. coli* strains which cause diarrhoreal disease are recognised. They are categorised on their virulence properties, mechanism of pathogenicity, clinical syndromes, and distinct O:H serogroups. They include: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAggEC), and enterohaemorrhagic *E. coli* (EHEC) (Doyle *et al.*, 1997). Many strains of *E. coli* from the EHEC group are capable of producing shiga toxin and are grouped as shiga toxin producing strains of *E. coli* (STEC) (Desmarchelier and Grau, 1997). This group has also been referred to in the literature as VTEC (verotoxigenic/verocytotoxigenic) or SLTEC (shiga-like-toxin-producing) strains of *E. coli.*

Traditionally much of the work on *E. coli* centred on its role as an index organism for enteric pathogens, such as *Salmonella,* and as an indicator of recent faecal contamination. However some strains were considered pathogenic as early as the 1900s when *E. coli* was associated with white scours in calves (Desmarchelier and Grau, 1997). By the mid-1940's, the role of *E. coli* as an enteropathogen was firmly established (ICMSF, 1996). There is a substantial literature on the pathogenicity, characteristics and

epidemiology of diarrhoeagenic *E. coli*. Several authors have reviewed this information (ICMSF, 1996; Desmarchelier and Grau, 1997; Doyle *et al.*, 1997).

1.1.1 A foodborne pathogen

Presently, four groups of pathogenic *E. coli* have been associated with foodborne illness: EPEC, ETEC, EIEC and EHEC. The extent of their involvement in foodborne illness throughout the world is difficult to assess, mainly because the procedures for their isolation and characterisation are not suited to routine food testing and few laboratories are equipped for their identification (ICMSF, 1996). Buchanan and Doyle (1997) consider the unusually virulent EHEC strains of *E. coli* are more significant than other well recognised foodborne pathogens due to their low infective dose, unusual acid tolerance, severity of consequence for all age groups and their apparently inexplicable association with ruminants. McClure and Hall (2000) challenge this general consensus and point out that survival studies of *E. coli* more often than not use a single strain, the O157:H7 serovar (*see* Section, S., 1.1.2), and fail to make comparisons to other *E. coli* strains. The work of Brown *et al.* (1997) highlights that 'unusual acid tolerance' is not unique to EHEC strains.

1.1.2 Serovar O157:H7

One serotype of *E. coli*, the STEC strain O157:H7, is considered the predominant pathogen in the EHEC group and the strain most frequently associated with human infections worldwide (Feng, 1995). It is known as an important cause of bloody diarrhoea (haemorrhagic colitis, HC) and renal failure (haemolytic uremic syndrome, HUS) (Doyle, 1991). Several severe life-threatening complications of HC can occur, of which HUS is the most common. Over half of HUS patients require dialysis and the mortality rate is 3-5% (Buchanan and Doyle, 1997).

E. coli O157:H7 achieved prominence when it was linked epidemiologically to inadequately cooked ground beef in sandwiches (Riley *et al.*, 1983; Wells *et al.*, 1983). Other foods of bovine origin epidemiologically linked to outbreaks of *E. coli* O157:H7 include hamburger patties (Willshaw *et al.*,

1994; CDC, 1996; CDC, 1997); dry-cured salami (CDC, 1995); cooked meat (WHO, 1997), pasteurized milk (Upton and Coia, 1994) and raw milk (Anon., 1986; Martin *et al.*, 1986; Borczyk *et al.*, 1987; Keene *et al.*, 1997). *E. coli* O157:H7 has also been implicated in outbreaks involving foods other than animal proteins, ranging from fresh vegetable produce through to highly acidic foods. This review, however, focuses on the relevance and impact of pathogenic *E. coli* on the meat industry.

1.1.3 Pathogenic *E. coli* and meat

Pathogens such as *E. coli* O157:H7 are an obvious concern to the meat industry as foods of bovine origin have been implicated as the principle vehicle in disease outbreaks. In most cases involving meat, evidence suggests the product was undercooked (Doyle, 1991). Cattle (Chapman *et al.*, 1993), in particular dairy cattle (Borczyk *et al.*, 1987; Padhye and Doyle, 1992; Zhao *et al.*, 1995; Wilson *et al.*, 1997), calves (Ørskov *et al.*, 1987; Rahn *et al.*, 1997), and unpasteurised milk (Borczyk *et al.*, 1987; Padhye and Doyle, 1991) have been identified as reservoirs of *E. coli* O157:H7. A prevalence survey of STEC in Australian farm animals (cattle, calves and sheep) is currently being undertaken (Desmarchelier, 1997). Preliminary findings report isolations of STEC from 10% of faecal samples with higher isolations from calves (14%) and sheep (19%) than cattle (1.8%). The STEC isolates belonged to a variety of serotypes. 13% of the isolates were of the serotype O157.

Raw foods of bovine origin are likely to be vehicles of *E. coli* O157:H7 through faecal contamination during slaughter, milking and processing procedures (Doyle, 1994). The same rationale applies to other pathogenic *E. coli.*

Vanderlinde *et al.* (1998) reported no significant difference in the distribution of numbers of *E. coli* on beef carcasses between Australia and the United States (based on the data of McNamara, 1995). From the same Australian survey of beef carcasses from 49 meat works commissioned by the Meat Research Corporation, *E. coli* O157:H7 was recovered from 4 of 1057 (0.38%) carcasses (CSIRO, 1996). A similar incidence of *E. coli* O157:H7 was found in a US baseline survey with recovery of this strain

from 4 of 2,081 (0.19%) steer and heifer carcasses (USDA, 1994). Heuvelink *et al.* (1998) examined the occurrence of *E. coli* O157 VTEC strains in Dutch food-producing animals and reported 10% (n=540) of adult cattle and 0.5% (n=397) of veal calf carcasses as positive. Chapman *et al.* (1993) isolated *E. coli* O157 from 84 (4%) of 2103 bovine rectal swabs from an abattoir in England. *E. coli* O157 was also isolated from 30% of the carcasses of these rectal swab positive animals, and from 8% of non rectal swab positive animals, thus highlighting the dangers of cross contamination.

Contamination of the carcass may result in contaminated products reaching the consumer. Comminuted products in particular pose a hazard as surface contaminants become evenly distributed throughout the product and can also be transferred to processing equipment. A study conducted by Farrell *et al.* (1998) demonstrated that *E. coli* O157:H7 in artificially contaminated ground beef can be readily transferred to the meat-grinder surface, thus providing opportunity for cross-contamination of products. Several surveys have reported pathogenic *E. coli* in ground beef and other ground meats, as well as raw meat products. Doyle and Schoeni (1987) surveyed retail meats from grocery stores in Madison, USA, and reported *E. coli* O157:H7 in 3.7% of ground beef samples. Similarly, Padhye and Doyle (1991) reported 2.8% of ground beef samples to be positive for *E. coli* O157:H7 in a survey of the same area. From several surveys conducted in the Netherlands, Heuvelink and co-workers have reported *E. coli* O157 VTEC strains in a variety of meats, including raw minced beef and other raw beef products, obtained from retail outlets and butcher shops (Heuvelink *et al.*, 1996; Heuvelink *et al.*, 1999). Interestingly, the 1999 study reported 1.1% (n=571) of raw minced beef samples to be positive for *E. coli* O157 VTEC, whereas the 1996 study reported 0% (n=1000). However, Heuvelink *et al.* (1996) acknowledge that the cultural methods employed in the early surveys of that study had some limitations.

1.1.4 Significance of pathogenic *E. coli* to the Australian meat industry

The strain O157:H7 dominates the food microbiology literature. This is partly because it is the only serotype which is routinely tested for,

especially in North America (Acheson *et al.*, 1996). Large outbreaks due to this strain, involving deaths, were well documented and received significant media coverage. Therefore the majority of recent research has focused on this strain. Despite the focus on *E. coli* O157:H7, there have been many well documented outbreaks of foodborne disease attributable to non-O157:H7 STEC strains (Johnson *et al.*, 1996). In Australia *E. coli* O157:H7 is not the predominant pathogen associated with foodborne disease. Other serotypes are more common and the pattern of EHEC serotypes in the human and animal population in Australia differs from countries in the Northern Hemisphere (Desmarchelier, 1997). An outbreak involving one death in Australia in 1995 of the serotype *E. coli* O111 was attributed to consumption of mettwurst, a fermented comminuted meat product (Anon., 1995).

The Australian meat industry and government agencies have been aware of increasing problems presented by *E. coli* O157:H7 in the Northern Hemisphere and have taken measures proactively to protect their meat supply and trade (Desmarchelier, 1997). These measures include funding of research in areas such as food production, animal and human health, and the development of diagnostic methods.

As mentioned previously, much of the work on pathogenic *E. coli* has centred on their pathogenicity, characteristics and epidemiology. The concerns of the meat industry, however, centre mainly on the microbiological quality of meat and meat products and prevention strategies for microbial contamination and proliferation, particularly that of pathogens. Predictive modelling has been proposed as a means of partially fulfilling these needs (*see* S. 1.5).

However, before predictive modelling and its application to the meat industry are considered it will be helpful to discuss the growth responses of bacteria to their environment, and meat as an environment for microbial growth.

1.2 ENVIRONMENTAL PARAMETERS AND MICROBIAL GROWTH

A number of environmental parameters influence the growth, death and survival of microorganisms. Limits are imposed on microbial growth in foods by a range of intrinsic and extrinsic parameters. Intrinsic parameters are those inherent in the food ecosystem and include: pH, a_w, nutrient content, oxidation-reduction potential (Eh), antimicrobial constituents and biological structures such as seed testa, animal hides and egg shells. Extrinsic parameters are those properties of the storage environment that affect the food and microorganisms and include: temperature, relative humidity and gaseous atmosphere (Jay, 1986). For each environmental parameter bacteria have an upper and lower limit for microbial growth, at which and beyond growth rate equals zero, as well as an optimum, where growth rate is maximal.

Of the many variables listed above, three are considered to have a primary influence on microbial growth in fresh meats. They are temperature, a_w and pH (lactic acid).

1.2.1 Temperature

Temperature is considered the most important environmental influence on the growth and survival of microorganisms in fresh foods due to the significant role temperature plays in biochemical reactions. Growth rate increases from the minimum temperature for growth with increasing temperature up to an optimal temperature where growth is fastest. The response is curvilinear. At temperatures higher than the optimum, growth rate declines rapidly with increasing temperature until the maximum temperature for growth is reached. The region above the optimum temperature for growth, i.e. from optimum to maximum, is much narrower than the region below the optimum, i.e. from the optimum to the minimum.

Temperature interacts with pH to affect the growth rate and range of microorganisms. a_w also acts synergistically with temperature to affect the cardinal temperature values for growth, i.e. the maximum, minimum and optimum values. Decreasing the a_w of the environment leads to an

increase in the minimum temperature for growth. Previously it was supposed that bacteria grew over the widest a_w range at the optimum temperature for growth. However, studies of the growth/no growth boundary of *E. coli* (Salter, 1998) and *Listeria monocytogenes* (*L. monocytogenes*) (Tienungoon, 1999) show them to be most tolerant to a_w stress in the temperature range 20 to 25°C. These temperatures are within the 'normal physiological range' (*see* S. 1.3.1.) for these organisms.

1.2.1.1 *E. coli* and temperature

E. coli, like most bacteria, grows over a range of approximately 40°C. An Arrhenius plot of the growth of *E. coli* B/r as a function of temperature, *see* Fig. 1.1, shows a normal physiological range of 21 to 37°C, with a maximum temperature for growth of 48°C (adapted from Herendeen *et al.*, 1979). Krist (1997) and Ross (1997) reported a lower limit for the normal physiological range for *E. coli*, around 17°C. Ingraham and Marr (1996) reported a minimum temperature for growth of 8°C, and Shaw *et al.* (1971) determined the minimum temperature for growth of *E. coli* ML30 to be between 7.5 and 7.8°C. McMeekin *et al.* (1987) also reported the minimum for growth of a strain of *E. coli* to occur at 7.8°C, and reported no growth observed at 7.2°C after 30 days incubation.

Pathogenic strains of *E. coli* generally display similar growth characteristics to non-pathogenic *E. coli* in response to temperature (Salter *et al.*, 1998). The minimum temperature for growth of *E. coli* O157:H7 is approximately 8 to 10°C if other factors are non-limiting (Buchanan and Bagi, 1994). Conflicting information regarding the maximum temperature for growth of *E. coli* O157:H7 led Salter *et al.* (1998) to determine whether this serotype and other STEC strains can grow in the temperature region traditionally used in isolation of *E. coli* (44 to 44.5°C). They demonstrated that all STEC strains, including *E. coli* O157:H7 but with the exception of an *E. coli* O157:H-, grew at temperatures above 45°C and that many strains showed significant growth at 46 and 47°C. These results are consistent with those of (Palumbo *et al.*, 1995).

1.2.2 a_w

The term a_w is used as an index of the osmotic potential of foods or laboratory media and is defined as the ratio of the vapour over a food or medium at equilibrium with the atmosphere above it to the vapour pressure of pure water at the same temperature (Troller and Christian, 1978). It is generally accepted as a suitable indicator of the amount of water available to microorganisms for enzymatic and non-enzymatic reactions. a_w should not be confused with moisture content, a measure of the total amount of water in a food, as some of the water in a food is bound chemically and physically to the protein substrate and any salts and sugars that may be present and is thus not available for microbial growth (Doe *et al.*, 1998).

Unlike the role of temperature for fresh foods, a_w is considered the most important environmental influence on microbial growth and survival in dry foods (Doe *et al.*, 1998). Traditional techniques for the preservation of perishable food, such as salting and drying, manipulate the a_w of the food matrix. Changes in a_w can be achieved by the addition of humectants such as sugar or salt, as well as by drying or freezing to either retard microbial growth or induce microbial death. In the food industry, lowered a_w is often used in combination with other environmental parameters to preserve food. For example, reduced/lowered a_w is used in combination with pH in the manufacture of cheese and salamis.

Bacteria share a response curve for a_w and growth rate that is similar to that for temperature. Below the optimum a_w for growth, growth rate declines approximately linearly with decreasing a_w, and above the optimum the decline is also linear but with a steeper tangent. The rate of decline and the range of a_w over which the decline occurs varies between bacteria. As for temperature, the a_w range above the optimum in which growth occurs is narrower than that below the optimum.

Reduction in a_w also affects the duration of the lag phase. Generally, at a_w levels close to the optimum for growth rate the lag phase is at its shortest, and at levels close to the minimum for growth rate the lag phase is extended. Reducing the a_w to sub-optimal conditions may have an

immediate effect on the lag phase of some species. However, for other bacteria the lag phase may remain unaffected by a similar a_w change (Troller and Christian, 1978).

1.2.2.1 a_w and 'cell yield'

Yield is defined as the amount of dry cell weight of organisms produced per gram of carbon substrate metabolised (Neijssel *et al.*, 1996). 'Cell yield' in a bacterial culture may be inferred by measurements of reduced final optical density, i.e. the change in optical density (ΔOD), in substrate limited batch cultures under various growth conditions. For a_w, ΔOD remains high over most of the growth permissive range and declines to zero at the low a_w extreme (Krist *et al.*, 1998a).

The concept of a normal physiological range for temperature is discussed in S. 1.3.1. For temperature, this range can be visualised readily with Arrhenius plots. Ross (1997) noted a relationship between relative yield and temperature for *E. coli* M23 that closely paralleled the deviation in growth rate from that expected on the basis of a simple Arrhenius model. Relative 'cell yield' remained constant (around 1.0) for much of the growth permissive range and began to decline at temperatures which coincided with the boundaries of the normal physiological range for *E. coli* M23, i.e. 17 and 37°C.

Unlike the bacterial response to temperature, there is, as yet, no mechanistic basis to describe the bacterial response to a_w. However, Krist (1997) reported that the 'cell yield' response of *E. coli* SB1 for temperature and a_w to be similar, i.e. 'cell yield' is high over most of the growth permissive range and declined as growth limits were approached, indicating that the influence of a_w on microbial growth may be explained in terms of protein folding. As yet, it is not possible to relate 'cell yield' to a normal physiological range for a_w in the same way that Ross (1997) related 'cell yield' to the normal physiological range for temperature.

1.2.2.2 *E. coli* and a_w

E. coli is a non-halophilic organism, i.e. its growth is not dependent on, or accelerated by, high salt concentrations. *E. coli* falls in the mid range of the tolerance to low a_w exhibited by non-halophilic bacteria (Ingraham and Marr, 1996). The minimum a_w permitting growth of *E. coli* is 0.95, or about 8% sodium chloride (Troller and Christian, 1978). This minimum for growth is influenced by pH and temperature, with effects most marked at low temperatures and low pH (Desmarchelier and Grau, 1997). As mentioned previously, tolerance to a_w is maximal around 20 to 25°C.

1.2.3 pH

It is well established that most microorganisms grow best at pH values around 7.0 (6.6-7.5), while few grow below 4.0. Bacteria tend to be more fastidious in their relationships to pH than molds and yeasts, with bacteria pathogenic to humans being among the most fastidious (Jay, 1986). pH minima and maxima vary between microorganisms, and these values may be affected according to the type of acidulant and alkali in their environment.

Organic acids are often used as a means of food preservation. They are either directly added to products or are naturally present as the result of microbial metabolism in fermented foods. Acetic, propionic, lactic, sorbic and benzoic acids are widely used as food preservatives, and have pK_a values that are between pH 3 and pH 5 (Eklund, 1989). The presence of organic acids accentuates the effect of pH, but the magnitude of the effect is dependent on the type of organic acid and the pH of the substrate. Both the dissociated and undissociated forms of organic acids have inhibitory effects on bacterial growth, but the undissociated form is much more inhibitory, per mole, than the dissociated form (Eklund, 1989).

Lactic acid is one of the most abundant acids in nature and occurs in many foods obtained by fermentation. It is added to foods such as brined gherkins and olives, pickles, salads, dressings, some confectionery, dairy and meat products. In fresh meat lactic acid is naturally present as a result of the anaerobic breakdown of carbohydrates (Smulders, 1986).

The effect of pH on the growth of microorganisms is well described (Gould, 1989). The optimal pH for bacterial growth is species dependent and usually occurs in the middle of the pH range. Growth rate rapidly declines to zero near the maxima and minima. The region of fastest growth occurs over a range of pH surrounding the optimum, usually 1 to 2 pH units.

Other environmental parameters interact with pH to affect the growth rate and range of growth of microorganisms. In general, additional stresses will raise the minimum pH that permits growth. For example, decreasing a_w values will narrow the pH growth range over which microorganisms can grow (Mossel *et al.*, 1995). A specific example for *E. coli* is presented below.

1.2.3.1 *E. coli* and pH

E. coli is classified as a neutrophile with regard to pH. It is generally accepted that in laboratory media, *E. coli* grows in the range of pH 4.4 to pH 10 (Desmarchelier and Grau, 1997). However, Presser *et al.* (1998) have demonstrated a pH limit of 4.0. Growth rates for *E. coli* are maximal between pH 6.0 and 8.0, and are slower at half a pH unit or so beyond these limits (Ingraham and Marr, 1996). The minimum pH for growth of *E. coli* is dependent on the type of acid present and acid concentration. For example, Glass *et al.* (1992) demonstrated a pH minimum of 4.5 in HCl adjusted medium for a strain of *E. coli* O157:H7, but the strain could not grow at this pH in media with lactic acid as the acidulant. Abdul-Raouf *et al.* (1993) reported the relative inhibitory activity of organic acids in the order acetic≥lactic≥citric for *E. coli* O157:H7 in beef slurries. The pK_a values for these organic acids decrease in a corresponding order of 4.73 to 3.85 to 3.08 respectively (Clark, 1928).

Other parameters affect the minimum pH for growth. Presser *et al.* (1998) demonstrated the effect of reduced a_w, with NaCl as the humectant, at a temperature of 20°C, on the minimum pH at which growth of *E. coli* M23 occurred. The minimum pH for growth was 4.2 at a_w 0.990, but increased to 6.2 at a_w 0.955.

Enteric pathogens need to survive the gastric acidity barrier before they can infect and colonise the host. *E. coli* O157:H7 has been epidemiologically linked to outbreaks resulting from consumption of acidic food such as apple cider (Besser *et al.*, 1993) and mayonnaise (Weagent *et al.*, 1994). Studies have shown *E. coli* O157:H7 to be acid tolerant (Connor and Kotrola, 1994; Benjamin and Datta, 1995). Acid tolerance however is not a feature unique to O157:H7 strains. Benjamin and Datta (1995) reported a high level of acid tolerance in several non-O157 EHEC strains. A high degree of intrinsic acid tolerance was also observed by Brown *et al.* (1997) for a non pathogenic laboratory strain, *E. coli* M23, and a pathogenic H- strain of *E. coli* O157.

It is evident that the fate of microorganisms is affected by a complex interplay of environmental parameters. Consequently there are opportunities for control of microorganisms in foods by manipulation of these parameters.

1.3 PHYSIOLOGICAL RESPONSES TO CHANGES IN ENVIRONMENT

1.3.1 Temperature

The thermodynamic relationship between bacterial growth and temperature has been described by several models, which were reviewed by McMeekin *et al.*, 1993; pp 96-110. These models are based on an Arrhenius' rate equation for chemical reaction and "assume that growth rate is governed by a single rate-limiting enzyme catalysed reaction" (McMeekin *et al.*, 1993). The addition of thermodynamic terms to describe the effects of high and low temperature on such a rate-limiting reaction lead to a deviation from Arrhenius' straight line relationship, as is observed for the microbial growth rate response to temperature presented in Fig. 1.1.

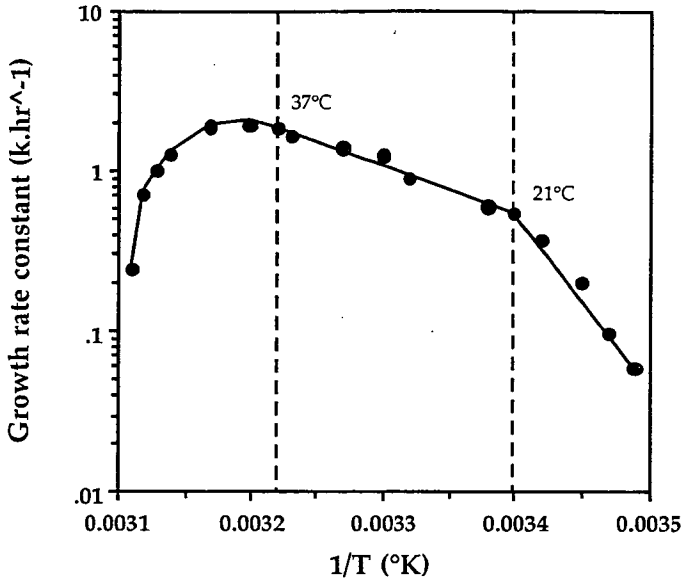

Figure 1.1: Arrhenius plot for growth of *E. coli* B/r adapted from Herendeen *et al.* (1979). The bounds of the normal physiological range are marked (- - -).

On an Arrhenius plot the response over the mid-range of temperature, termed the 'normal physiological range' for temperature, is linear. Approaching the maximum and minimum temperatures for growth, the asymptote deviates from Arrhenius kinetics and approaches vertical. These areas are termed the high and low ranges for temperature, with cessation of the response occurring at the maximum and minimum temperatures for growth. The slope of the linear region and cardinal temperatures for growth vary widely among species, however "the general form of the Arrhenius plot of growth rates is typical for all bacteria studied" (Ingraham and Marr, 1996).

Krist (1997) has demonstrated that the low temperature boundary at which deviation from linear Arrhenius kinetics occurs is not fixed. Altering the extracellular a_w conditions changes the temperature at which deviation occurs; as a_w conditions become more stringent the normal physiological range for temperature is narrowed.

1.3.2 Osmotic pressure

For bacteria to survive and grow they must be in osmotic equilibrium with their environment. Osmotic equilibrium in bacteria is achieved by developing positive turgor pressure such that the a_w of the cytoplasm equals that of the surrounding medium (Ingraham and Marr, 1996). Maintenance of turgor pressure is essential for growth and division of the cell (Ingraham and Marr, 1996). Gram positive bacteria generally maintain a higher turgor than gram negative bacteria due to their higher cytoplasmic concentration of solutes at equivalent osmotic pressures (Csonka, 1989; Patchett *et al.*, 1992; Ingraham and Marr, 1996).

Much of the literature on osmotic stress focuses on the effect of hyperosmotic shock. Reduced a_w is a common strategy employed in the food industry. When the a_w of the external medium is reduced, cells rapidly lose water, a process called plasmolysis (Sperber, 1983). Consequently the cytoplasmic volume of the cell is reduced and turgor pressure decreases. Hyperosmotic shock may render the cell dormant or lead to cell death unless the intracellular a_w is adjusted to restore turgor pressure and cytoplasmic volume to their pre-stress values.

Osmotic regulation, or osmoregulation, may be defined as "the active processes carried out by organisms to cope with osmotic stress" (Csonka, 1989). The semi-permeable lipid membrane of bacteria plays an important role in osmoregulation; for the efflux and influx of water, and the accumulation or exclusion of solutes. Due to the rigidity of the cell wall, bacteria cannot rely on passive water accumulation to regulate turgor pressure and instead respond to hyperosmotic stress by increasing the concentrations of a limited number of solutes (Csonka, 1989). Such solutes have been termed 'compatible' as they do not interfere with, nor are they inhibitory to, normal cellular metabolic processes.

Compatible solutes attract water, thereby restoring or partially restoring iso-osmotic conditions across cell membranes (Rockland and Beuchat, 1987), and also protect enzyme activity (Brown, 1976). Some compatible solutes are referred to as osmoprotectants due to their ability to palliate osmotic inhibition of growth (Csonka, 1989). Prominent compatible

solutes in bacteria include K^+ ions, glycine betaine, amino acids such as proline and glutamate, and sugars such as trehalose and glycerol. Numerous other compounds act as compatible solutes (*see* Csonka, 1989), with glycine betaine the most widespread (Imhoff, 1986). In the majority of non-halophilic bacteria osmoregulation is accomplished by amino acids (Rockland and Beuchat, 1987). Foods can contain compounds which act as compatible solutes, chiefly betaine, proline and sources of choline (Gutierrez *et al.*, 1995). Complex laboratory media also contain compatible solutes; for example, yeast extract contains considerable amounts of glycine betaine, 1-3% by dry weight (Galinski, 1993). Compatible solutes may be transported from the environment or synthesised *de novo* in the cytoplasm (Gutierrez *et al.*, 1995). Compatible solutes are unable to cross cell membranes rapidly without the aid of transport systems (Csonka, 1989).

1.3.2.1 Gram-negative bacteria

Generally, gram-negative rods are most sensitive to reduced a_w and have minima for growth in laboratory media in the range 0.96-0.94 (Troller and Christian, 1978). Osmoregulation has been studied extensively for the gram-negative enteric bacteria *E. coli* and *Salmonella* Typhimurium (*S.* Typhimurium).

1.3.2.1.1 *E. coli* and *S.* Typhimurium

The primary response of *E. coli* and *S.* Typhimurium to hyperosmotic shock is accumulation of K^+, and this is the major mechanism of turgor regulation (Epstein, 1986). Intracellular K^+ is increased partly due to plasmolysis and partly due to active accumulation from the environment. The level of intracellular K^+ accumulated increases systematically with increasing external a_w to maintain positive turgor pressure (Ingraham and Marr, 1996). In *E. coli*, K^+ uptake is facilitated by two major transport systems, Trk and Kdp, and occurs via specific sites in the inner cell membrane (Rockland and Beuchat, 1987). The Trk system is constitutive with a low affinity for K^+ (Epstein, 1986), and is used when the cell is not osmotically stressed, but increases K^+ uptake in response to osmotic stress. The Kdp system is inducible with a high affinity for K^+ (Epstein, 1986). It

operates under conditions of low a_w and is used when the primary Trk system cannot satisfy K^+ requirements. Homologs of Trk and Kdp have been demonstrated in *S.* Typhimurium (Parra-Lopez *et al.*, 1994, cited in Csonka and Epstein, 1996). To maintain electroneutrality, increasing intracellular K^+ is balanced by an increase in its counterion glutamate (Botsford *et al.*, 1994; Ingraham and Marr, 1996).

The secondary response of *E. coli* and *S.* Typhimurium to hyperosmotic shock is replacement of ionic solutes, K^+ and glutamate, by compatible solutes such as trehalose (by synthesis) or glycine betaine (by transport) (Ingraham and Marr, 1996). For *E. coli*, synthesis of trehalose is governed by the expression of genes (Giaever *et al.*, 1988). Trehalose is transported in at least two different ways for *S.* Typhimurium, via II^{Man} of the PTS and via the galactose permease, if the latter transport system is synthesized constitutively (Postma *et al.*, 1986). The rate of trehalose synthesis is dependent on the pre-history of the organisms, with cells from stress free environments first having to express the appropriate genes (Gutierrez *et al.*, 1995). *E. coli* is unable to synthesise glycine betaine *de novo* and relies on transport systems to accumulate it in the cytoplasm. Selected strains can synthesise glycine betaine from exogenously supplied precursors choline and glycine betaine aldehyde (Strøm *et al.*, 1986). *E. coli* and *S.* Typhimurium both possess two major betaine transport systems, ProP and ProU, which are low and high affinity systems respectively (Cairney *et al.*, 1985a; Cairney *et al.*, 1985b). In non-stressed cells only the semi-constitutive ProP is present, with ProU induced in response to osmotic stress (Gutierrez *et al.*, 1995). Both transport systems also mediate the uptake of proline (Csonka, 1989).

1.3.2.2 Gram-positive bacteria

Gram-positive bacteria maintain a higher turgor pressure than gram-negatives and possess constitutive and inducible mechanisms for osmoregulation. Constitutive mechanisms for the accumulation of compatible solutes may play a role when the organism is not osmotically stressed and contribute to the osmotolerance usually displayed by gram positive organisms.

1.3.2.2.1 *S. aureus*

Staphylococcus aureus (S. aureus) is the most halotolerant, non-halophile eubacterium (Gutierrez *et al.*, 1995) and has a minimum a_w for growth as low as 0.86 (Scott, 1953). The minimum a_w for growth is influenced by the controlling solute, with NaCl and sucrose tolerated better than glycerol (Ash, 1997). *S. aureus* naturally contains high levels of cytoplasmic K^+, a contributing factor to its osmotolerance, the intracellular concentration of which changes little with increasing osmotic strength of growth media (Graham and Wilkinson, 1992). Therefore, in contrast to enteric bacteria, K^+ levels are less important for osmoregulation in *S. aureus* (Graham and Wilkinson, 1992). This implies accumulation or synthesis of some other form of compatible solute in response to osmotic shock (Armstrong-Buisseret *et al.*, 1995).

Glycine betaine is accumulated to high levels by two sodium dependent transport systems that can be differentiated on the basis of their affinity for glycine betaine and their activation by osmotic pressure (Pourkomailian and Booth, 1992). The high affinity system is relatively independent of osmotic pressure, therefore glycine betaine accumulation occurs even under conditions of low osmolality. The low affinity system is inducible by osmotic pressure (Pourkomailian and Booth, 1992).

The osmoprotective nature of choline for *S. aureus* was first demonstrated by Graham and Wilkinson (1992). *S. aureus* can actively transport choline into the cell in response to osmotic shock, then metabolises it into glycine betaine (Kaenjak *et al.*, 1993). Choline uptake occurs via an inducible, sodium dependent transport system (Kaenjak *et al.*, 1993), therefore gene expression is necessary following osmotic shock (Armstrong-Buisseret *et al.*, 1995).

The most common mechanism for accumulating proline in osmotically inhibited bacteria is synthesis (Rockland and Beuchat, 1987), however *S. aureus* accumulates high intracellular concentrations of proline during osmotic stress only by enhanced transport (Anderson and Witter, 1982). Two sodium dependent transport systems for proline have been described by Townsend and Wilkinson (1992); a high affinity system for

scavenging exogenous proline and a low affinity system for osmoregulation. Neither system requires induction by proline or hyperosmotic shock, suggesting new protein synthesis is not necessary for rapid proline uptake and that hyperosmotic shock activates a pre-existing transport system (Townsend and Wilkinson, 1992). Proline uptake occurs more rapidly than glycine betaine (Kaenjak and Wilkinson, 1991), suggesting that proline may be very important in the initial response to osmotic stress in *S. aureus* (Townsend and Wilkinson, 1992).

1.3.2.2.2 *L. monocytogenes*

L. monocytogenes can grow over a wide range of salt concentrations and, as in other bacteria, this adaptability appears to be achieved by the ability of the organism to accumulate intracellular solutes (Patchett *et al.*, 1992). *L. monocytogenes* accumulates K^+, glycine betaine and glutamate (Patchett *et al.*, 1992) and, when grown in a complex medium, amino acids and peptides (Amezaga *et al.*, 1995). Patchett *et al.* (1992), when investigating the effect of increasing NaCl concentration on the intracellular solute pools of *L. monocytogenes*, found elevated levels of glycine betaine and K^+ after exposure to 5% (wt/vol) NaCl. Exposure to 7.5% (wt/vol) increased the levels further. Ko *et al.* (1994), through NMR studies, found no evidence for any endogenously synthesised glycine betaine, therefore *L. monocytogenes* relies solely on an exogenous supply. The glycine betaine transport system of *L. monocytogenes is* constitutive or, at most, weakly induced by NaCl, with cells transporting betaine 200-fold faster at a salt concentration of 4% compared with no salt (Ko *et al.*, 1994). *L. monocytogenes* lacks a proline uptake system and unlike *S.* Typhimurium (Cairney *et al.*, 1985a; Cairney *et al.*, 1985b) and *E. coli* (May *et al.*, 1986), the betaine system cannot take up proline. *L. monocytogenes* also possesses a constitutive high-affinity uptake system for exogenously supplied carnitine, which may also play a role in maintaining turgor pressure in the absence of osmotic stress (Verheul *et al.*, 1995).

1.3.3 pH

Bacteria respond to adverse pH in their environment by altering their cytoplasmic pH, denoted pH_i. This is achieved by three progressively more stringent mechanisms: a homeostatic response, the acid tolerance response or synthesis of acid shock proteins (Montville, 1997). The pH of the external environment has to change by several pH units before pH_i is affected. Homeostasis breaks down only at the more extreme conditions. For neutrophiles such as *E. coli*, pH_i values are in the range 7.5 to 8.0 (Booth, 1985). Although alkaline internal pH values are optimal for growth, *E. coli* has a marked tolerance to reductions of pH_i of up to one unit (Booth and Kroll, 1989).

The mechanisms of pH homeostasis are incompletely understood, but the cells' ability to regulate its internal pH is not a passive process. In essence, regulation of cytoplasmic pH implies control over the permeability of the cell membrane to protons. It is generally accepted that this is achieved by control of the activity of ion transport systems which facilitate proton entry. Possible mechanisms for cytoplasmic pH regulation include cytoplasmic buffers, biochemical production of acids and bases, active transport of H^+ (or its equivalent OH^-), and these are reviewed in Booth (1985). The rapid recovery of internal pH upon a shift of external pH by one or two units indicates that some aspects of pH homeostasis must be constitutive, although some inducible components exist as well (Slonczewski and Foster, 1996).

1.4 MEAT AS AN ENVIRONMENT FOR MICROORGANISMS

Meat is a highly favourable environment for the proliferation of microorganisms because it contains nutrients necessary for the growth of yeasts, bacteria and moulds. The pH of meat is within the growth range of most organisms, a_w is generally high and oxygen/redox conditions at the surface are suitable for growth (Jay, 1986).

The a_w of lean muscle tissue is around 0.990, corresponding to a water content of 74 to 80% (Jackson *et al.*, 1997). Typically pH values are in the range 5.5-6.5 (Dainty and Mackey, 1992). Differences in pH occur between

carcasses and muscle types (Gill and Newton, 1978). At the time of death the pH of muscle tissue is 7.0-7.2; the final pH falls to a level of 5.5-5.7 due to glycolysis (Gill and Newton, 1978). The lactic acid content of fresh meat is dependent on the energy stores of the animals at the time of slaughter, with beef typically containing $9g.Kg^{-1}$ lactate (100 mM) (Smulders, 1986). Grau (1981) determined the L-lactate content of beef muscle of pH 5.55 to be 115 to 145mM, and for muscle of pH 6.1, 65 to 105mM.

Because of its suitability as a growth medium, the nature and level of microbial contamination of meat has important consequences for public health, storage life and the type of spoilage likely to develop (Nottingham, 1982).

1.4.1 Meat microbiology and contamination

The microbiology of carcass meats is highly dependent on the conditions under which the animals are reared, slaughtered and processed (Nottingham, 1982). Slaughter animals are often asymptomatic carriers of a wide range of pathogenic microorganisms which, unless they are associated with particulate matter, are undetectable by visual inspection. Consequently great care must be taken at the level of primary production (abattoirs) to minimise these invisible hazards.

The microbiological quality of meat is determined by:

- the condition of the animal at slaughter;
- the spread of contamination during slaughter and processing; and
- the conditions encountered during storage and distribution, primarily temperature/time combinations (Nottingham, 1982).

Muscle tissue is generally regarded as sterile (Gill, 1982). In comparison, high levels of bacteria may be present on the external surfaces such as the hides, hooves and hair of red meat animals. Internal sources include lymph nodes (Jay, 1986), the gastrointestinal tract, respiratory tract, urine and milk (Nottingham, 1982). The hide is regarded as the origin of most contaminating microbiota of dressed red meat carcasses, with microbiota originating in soil, water, vegetation and faecal material (Newton *et al.*, 1978). Hides with no visible evidence of faecal or other macro-contamination may carry a mesophilic microbial load as high as

5.10 $\log_{10}$ cfu.cm^{-2} (Bell *et al.*, 1997). The population and composition of the hide microbiota is influenced by environmental conditions (Nottingham, 1982).

In beef carcass dressing procedures, faecal contamination is regarded as the major hazard (Gill *et al.*, 1996). Initially the tissue surface beneath the hide is bacteria free; however, once exposed, this tissue may become contaminated with bacteria from processing activities and the production environment (Jackson *et al.*, 1997). The process of hide removal exposes underlying tissue to potential contamination from contact between dirty and clean surfaces, airborne particles and aerosols (Nottingham, 1982). The evisceration process may also introduce bacteria from piercing of the intestinal tract, contamination during removal of abdominal contents and cross-contamination from handling (Jackson *et al.*, 1997). *E. coli* generally comprise the greatest proportion of the total aerobic flora of the intestine (Nottingham, 1982), and is thus a potential contaminant.

1.4.2 Microbial proliferation on meat carcasses

Currently available dressing procedures inevitably result in contamination of the carcass surface, and it must be assumed that contaminants will include spoilage and potentially pathogenic bacteria (Gill and Phillips, 1990). After dressing, red meat carcasses typically carry between 10^2 and 10^4 bacteria.cm^{-2}; mesophiles are predominant in the initial flora and originate from the intestinal tract and external surfaces of the live animal (Dainty and Mackey, 1992; Johnston and Tompkin, 1992). Cold-tolerant bacteria are also present, usually only to about 10^1.inch^{-2} (Johnston and Tompkin, 1992), but will outgrow the mesophilic biota at chill temperatures (Gill and Newton, 1978).

The most important factor affecting the microbial flora of meat is temperature; this determines whether microorganisms increase or decrease and influences the nature of the flora which becomes dominant (Nottingham, 1982). The surface of a freshly dressed carcass is wet and warm, 25 to 30°C, and deep body temperatures are 38 to 40°C (Eustace, 1981). Thus the carcass surface is an ideal environment for near maximum growth rates of mesophiles, and microbiological concerns centre on

microbial proliferation (Nottingham, 1982). The most effective pathogen-minimisation strategy post carcass dressing is subsequent continuous hygiene (Dorsa, 1997), and provision of conditions to retard microbial growth. 7°C is generally accepted as the maximum temperature for ensuring that mesophilic, enteric pathogens will not proliferate (Smith, 1985). Consequently cooling of carcasses to this temperature within a specified time has become a feature of many meat hygiene regulations. There is no absolute hygienic need to reduce the internal carcass temperature below this level (Gill, 1998). As mentioned previously, internal tissue is essentially regarded as sterile and it is the surface microbiota that are required to be hygienically controlled by the carcass cooling process.

In practice, cooling of carcasses after dressing takes place under a variety of conditions depending on the facilities available and the storage life required (Nottingham, 1982). During the past decade most slaughtering plants in North America adopted the practice of intermittently spraying carcasses with water during the first few hours of chilling (Gill, 1998). Beef sides are cooled overnight and then graded on a sales floor maintained at 2 to 4°C. This technique of spray chilling, i.e. using cold misted water, has been demonstrated to minimise weight loss (Gigiel *et al.*, 1989). Loss of carcass weight is considered economically undesirable. a_w is not a determining factor in this process due to lack of significant evaporative water loss, i.e. a_w remains high. In contrast, Australian meat works employ air drying in blast freezers where the rate of cooling is determined by the temperature, relative humidity and air velocity in the meat cooler. A typical weekend cooling pattern in an Australian abattoir employs fan speeds set at 110% (~1m.sec^{-1}) at 8.5°C for the first 8 hours of chilling, 60% at 9.5°C for the following 12 hours and 40% at 9.5°C for the remainder of the time period (Salter, 1998). Overnight chilling practices are structured in the same way over a shorter time period. Rapid chilling by blast freezing of carcasses reduces meat temperature and dries the meat surface (Eustace, 1981; Salter, 1998).

1.4.3 a_w and carcasses

After temperature, availability of water is perhaps the most important requirement for growth of microorganisms on meat (Lawrie, 1991). a_w may play an important role in the long-term chilled storage of carcass meats (Troller and Christian, 1978). Immediately after dressing, the evaporation of water from warm carcasses can dry carcass surfaces sufficiently to inhibit bacterial proliferation (Nottingham, 1982). Scott and Vickery (1939), in their study on the effect of cooling rate, humidity and air velocity on the microbial population on beef during chilling and storage, showed that the microbiological condition of cooling beef carcasses was largely dependent upon the extent to which the surface of the carcass dried during cooling. They observed a reduction in the growth of psychrotrophic bacteria on beef due to decreased a_w from surface drying. They also identified important differences between the cooling and storage phases of dressed carcasses. The rate of desiccation of the surface tissues depends on the difference between the rate of evaporation and diffusion of moisture from the deeper layers of tissue. Drying of the carcass surface occurs mainly during the early part of cooling when the surface is warmer than the air. During storage, moisture will diffuse through to the surface at a rate exceeding that of evaporation, and thus the surface a_w is increased. The adverse effects on the bacterial flora of desiccation and cold reinforce each other; the faster the rate of cooling of tissue surfaces the less desiccation required to prevent microbial proliferation (Nottingham, 1982).

Carcass surface temperature is easily monitored, however measurement of a_w is more problematic. Salter (1998) successfully employed a simple and rapid technique to measure a_w directly at the carcass surface. Surface tissue samples were excised using a skin-grafting scalpel and a_w measured using a dew point a_w meter. Using this technique he was able to map a_w over the carcass surface and at different stages during chilling. a_w profiles on 6 carcasses at one abattoir appeared to follow a similar pattern of large fluctuations over the first 20 hours of chilling, with values falling as low as a_w 0.929 at the rib site and 0.942 at the brisket site, thereafter a_w remained relatively high. The increase in a_w after 20 hours coincided with carcass surfaces reaching temperatures similar to that of the air. As noted above,

water will diffuse from the deeper tissues to the surface once evaporative loss ceases due to temperature equilibrium.

1.5 PREDICTIVE MICROBIOLOGY AND MODELLING

Predictive microbiology is based on the concept that microbial responses to environmental conditions are reproducible. These responses can be quantified and expressed in the form of mathematical models which allow prediction of microbial responses, namely growth and decline, to new environmental conditions based on past observations. Microbial responses to the main controlling factors of temperature, pH, gaseous atmosphere, chemical preservatives, and a_w can be modelled individually or concomitantly (McMeekin *et al.*, 1993).

Predictive modelling is not a recent concept. The canning industry developed models for the calculation of thermal death times in the 1920's (Whiting and Buchanan, 1997). Scott (1937), from his study of the relationship between temperature and bacterial growth on ox muscle, also recognised the potential value of collecting bacterial kinetic data for shelf life determinations. The application of mathematical modelling techniques to the growth and survival of microorganisms in foods, however, did not receive wide attention until the 1980's (Ross and McMeekin, 1994).

Predictive modelling has developed as an adjunct to traditional techniques to determine the microbiological quality of food. This is in response to increased consumer awareness and requirements for a safe and wholesome food supply, recognition of the limitations of traditional methods and increased access to computers and improved software (McMeekin *et al.*, 1993). Many traditional techniques have unsatisfactory detection limits, lengthy time requirements and little predictive value. This leads to the provision of results retrospectively; an obvious failing as consumer markets demand minimally processed and ready-to-eat foods. Such foods often have short shelf lives. Although there have been many advances in rapid testing techniques, they are usually expensive and may require specialised equipment and expertise beyond the capabilities of many food testing laboratories. McMeekin *et al.* (1993) proposed that

predictive modelling may partially fulfill the need for rapid and cost effective microbiological assurance of quality and safety.

Predictive modelling has been traditionally divided into two main categories; kinetic modelling and probability modelling. Kinetic models are concerned with the extent and rate of growth or death of microorganisms; probability models are concerned with predicting the likelihood of an event occurring within a given time period (Ross and McMeekin, 1994). Integration of the two modelling approaches has resulted in the development of a newer class of probability models, interface models, which model the growth/no growth boundary (Ratkowsky and Ross, 1995). These models enable predictions of the probability of growth given an infinite time limit, i.e. whether growth is possible under the defined conditions or not, and model the interactions between factors on tolerance ranges discussed earlier.

1.5.1 Kinetic models

Kinetic models consider foods as nutrient rich substrates that are exploitable by microorganisms and approximate 'batch culture' conditions. Intrinsic and extrinsic environmental parameters of the food system then dictate the rate and extent of microbial proliferation. A detailed knowledge of the growth responses of microorganisms to those environmental parameters should enable prediction of the rate and extent of microbial growth in foods during processing, distribution and storage by monitoring the environment presented to the organism in the food during those operations (Ross and McMeekin, 1994). Growth rate, as affected by environmental parameters, may be modelled and then used to make predictions based on exponential growth. Alternatively, a sigmoid function (Gibson *et al.*, 1988) may be fitted to an observed population growth curve and the effect of environmental factors on the values of the parameters of the fitted sigmoid curve are modelled (Ross and McMeekin, 1994). For both approaches models are constructed by following the increase in numbers or biomass of the organism. A range of levels and combinations of environmental factors of interest are used to provide information on the kinetics of growth (Ross and McMeekin, 1994).

Four main categories of kinetic models are recognised:

- Belehrádek, or square-root, type models,
- Arrhenius-type models,
- Modified Arrhenius, or Davey models,
- Polynomial, or Response Surface models.

Their development has been reviewed by McMeekin *et al.* (1993). Many publications exist which compare the different types of kinetic models (Adair *et al.*, 1989; Heitzer *et al.*, 1991; Ratkowsky *et al.*, 1991; Zwietering *et al.*, 1991). Results to date are inconclusive regarding the superiority of any particular model type. Other aspects of modelling seem to have more effect on model performance than the model itself. Quality and quantity of data is important, as is the modelling process employed (McMeekin *et al.*, 1993). Researchers in different laboratories tend to employ model types based on personal preference and the extent of their computing facilities.

1.5.1.1 Square-root type models

The square-root type model proposed by Ratkowsky *et al.* (1982) describes a linear relationship between the square root of growth rate and temperature for bacterial cultures grown between the minimum and optimum growth temperatures and takes the form:

$$\sqrt{r} = b(T-T_0) \qquad \text{Eqn. (1)}$$

where r = growth rate, b = slope of the regression line, T = temperature and T_0 = conceptual minimum temperature for growth which is of no metabolic significance. Data is plotted as the square root of growth rate versus temperature and extrapolation of the regression line from this plot yields the theoretical minimum growth temperature value (T_0) where the regression line intersects the x axis.

Extension of Eqn. (1) to include growth data between the optimum and maximum temperature for growth led to development of a non-linear equation which covers the full biokinetic temperature range:

$$\sqrt{r} = b(T-T_{min})\{1-\exp[c(T-T_{max})]\} \quad \text{Eqn. (2)}$$

where T_{min} (equivalent to T_0 in Eqn. (1))[1] and T_{max} are the minimum and maximum temperatures, respectively, and *c* is an additional parameter to enable the model to fit the data for temperatures above the optimum (Ratkowsky *et al.*, 1983). Eqn. (2) is often referred to in the literature as the 'four parameter square root model for temperature'.

The square-root type model is not limited to descriptions of the bacterial growth response to temperature. Other environmental parameters can be modelled in combination with temperature. For example, the microbial growth rate response to a_w is linear over a range of a_w. Thus, the *b* term in Equations (1) and (2) is the only parameter that varies with a_w. Substituting this term with one that accounts for the effect of a_w, Eqn (2) can be extended to:

$$\sqrt{r} = d\sqrt{(a_w-a_{wmin})}\,(T-T_{min})\,\{1-\exp[c(T-T_{max})]\} \quad \text{Eqn. (3)}$$

where *d* = fitted parameter , a_w = water activity and a_{wmin} = theoretical minimum a_w for growth and all other terms are as defined previously (McMeekin *et al.*, 1993). Miles *et al.* (1997) extended Eqn. (3) to include a term to describe the effects of super-optimal a_w:

$$\sqrt{r} = b\sqrt{(a_w-a_{wmin})}\{1-\exp\{d(a_w-a_{wmax})]\}\,(T-T_{min})\,\{1-\exp[c(T-T_{max})] \quad \text{Eqn. (4)}$$

where *b*, *c* and *d* are fitted parameters, a_{wmax} = theoretical maximum a_w for growth and all other terms are as defined previously.

Similarly, the response to pH can be described by a square-root type model. Presser *et al.* (1997) developed a model which described the effects of pH and lactic acid by inclusion of novel terms for the inhibition due to

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[1] The notation was changed from T_0 to T_{min} as it was found that users interpreted growth at T_0 as growth at 0°C

the presence of hydrogen ions, undissociated and dissociated lactic acid species:

$$\sqrt{r} = C\sqrt{(a_w - a_{wmin})}(T - T_{min})\left(1 - \frac{10^{pHmin}}{10^{pH}}\right)\left(1 - \frac{[LAC]}{[U_{min}](1+10^{pH-pKa})}\right)\left(1 - \frac{[LAC]}{[D_{min}](1+10^{pH-pKa})}\right)$$

Eqn. (5)

where C = fitted parameter, pH_{min} = theoretical minimum pH for growth corresponding to the maximum theoretical hydrogen ion concentration, [LAC] = lactic acid concentration (mM), U_{min} = minimum concentration of undissociated lactic acid which prevents growth, D_{min} = minimum concentration of dissociated lactic acid which prevents growth, pKa = pH at which levels of undissociated and dissociated lactic acid are in equilibrium, reported to be 3.86 (Budavari, 1989), and all other terms are as defined previously.

Square-root type models, initially used in modelling the bacterial growth response to temperature, can be extended to include the pH (lactic acid) and a_w response. It should be noted that the multiplication of the terms as described above presupposes no synergism between parameters.

1.5.2 Predictive microbiology and the food industry

In the early 1990's, concerns regarding the true potential of predictive microbiology were expressed in the literature. They covered such areas as the effect of the initial numbers of contaminant microorganisms; identification of suitable models; the inherent distrust of empirical models; the effect of non-constant conditions; the complexity of food systems; the inherent variability of biological systems; and collection of appropriate environmental information (Ross *et al.*, 1993). These concerns have been reviewed (McMeekin and Ross, 1993; McMeekin *et al.* 1993).

Ross *et al.* (1993) stated that "realisation of the potential of predictive modelling is dependent on a conscientious and rigorous approach to data collection and modelling, ingenious solutions and strategies for the application of that data and models, and a willingness of industry to embrace the premises upon which predictive microbiology is based and to

trial the approach in their food systems". Application of such reasoning had already resulted in strong evidence supporting the use of predictive models in foods (Pooni and Mead, 1984; Gibson *et al.*, 1988; Hudson and Mott, 1993), and predictive models have also been employed successfully in the meat industry, particularly by Gill and co-workers in New Zealand. However there are documented exceptions (Walls and Scott, 1996; Walls *et al.*, 1996; Dalgaard and Jørgensen, 1998).

1.5.2.1 Meat and meat products

Several studies have been undertaken to develop and apply predictive models through the process of temperature function integration, TFI, i.e. calculation of bacterial growth from product temperature histories and models relating temperature to bacterial growth (Table 1.1). TFI has been used to assess the hygienic adequacy of meat processing in studies ranging from laboratory scale experiments to commercial operations and encompasses offal chilling procedures, meat handling processes, meat thawing procedures and storage and distribution practices.

The authors listed in Table 1.1 have developed and/or validated temperature dependent models based on square-root kinetics for mesophilic enteric organisms such as *E. coli* and *Salmonella*.

Although the list in Table 1.1 is not exhaustive, collectively the publications illustrate the potential of models to predict accurately the behaviour of enteric organisms on meat when temperature is the sole factor controlling growth.

In general, acceptable prediction of the growth rate and limits of microorganisms in foods has been demonstrated by many researchers. However prediction of lag times has proved more problematic (Walls and Scott, 1996; Dalgaard and Jørgensen, 1998).

Table 1.1: Application of predictive models in the meat industry

Author	Model and/or performance evaluation undertaken
Gill (1984)	Development of anaerobic growth model for growth of *E. coli*, performance evaluation in offal cooling procedures.
Gill and Harrison (1985)	Performance evaluation of Gill (1984) model in offal cooling procedures.
Smith (1985)	Model of aerobic growth of coliforms on meat - implications for codes of practice (laboratory study).
Gill (1986)	As for Gill (1984).
Smith (1987)	Performance evaluation of Smith (1985) in raw blended mutton (laboratory study).
Mackey and Kerridge (1988)	Models for Salmonella in minced beef (growth rate and lag phase duration). Effect of inoculum size (laboratory study).
Gill *et al.* (1988)	Computer programs to evaluate process hygiene. Models for aerobic and anaerobic conditions, lag phase and growth rate, but no details given.
Lowry *et al.* (1989)	Aerobic models for *E. coli* lag phase duration and growth rate. Performance evaluation for meat thawing procedures.
Gill and Phillips (1990)	Gill (1984) model validated for offal cooling and Gill *et al.* (1988) model for carcass cooling. TFI criteria for carcass cooling.
Gill *et al.* (1991a)	Performance evaluation of Gill *et al.* (1988) for beef carcass cooling.
Gill *et al.* (1991b)	Performance evaluation as above for spray cooling of carcasses.
Reichel *et al.* (1991)	Performance evaluation as above and anaerobic model of Gill (1984) in hot boning process.
Gill and Jones (1992a)	Performance evaluation of aerobic *E. coli* model in cooling of pig carcasses.
Gill and Jones (1992b)	Performance evaluation of an anaerobic model of Gill (1984) in beef offal.
Gill *et al.* (1995)	Performance evaluation of aerobic and anaerobic model of Gill *et al.* (1988) for offal cooling.
Gill and Jones (1997)	Performance evaluation of *E. coli* model of Gill *et al.* (1988) for air-cooling of lamb carcasses and spray-cooling of pig carcasses.
Jericho *et al.* (1998)	Performance evaluation of aerobic and anaerobic model of Gill *et al.* (1988) for beef carcass cooling.

1.6 OBJECTIVES OF THIS THESIS

Pathogenic *E. coli* are of world-wide significance with an established record for disease outbreaks associated with foods of bovine origin. Although responsible for comparatively few outbreaks in Australia, this pathogen is none-the-less of concern to the Australian meat industry.

One objective of this thesis is to develop a new square-root type model for *E. coli* growth which incorporates parameters appropriate to conditions in meat. The performance of this model will be assessed by comparison of model predictions to data derived from laboratory inoculation studies in ground beef and to published data for growth of *E. coli* in liquid growth media and foods. The performance of the model will also be compared to other existing models for *E. coli* growth. For reasons discussed in S. 4.1.1.3, it is not possible to assess the performance of the *E. coli* model in industry. However, a surrogate organism may be used. Accordingly, a square-root type model for *Klebsiella oxytoca* (*K. oxytoca*) growth will be developed. Assessment of this model will not be undertaken by the candidate, however preliminary results from carcass chilling trials conducted by industry collaborators are considered.

Chilling regimes in Australian meat works reduce temperature rapidly and dry the carcass surface, thus lowering a_w. As discussed in S. 1.3, temperature and a_w significantly affect the kinetics and tolerance ranges of microbial growth. The lag phase is an area of interest for the meat industry, therefore the effects of abrupt shifts in temperature, pH and a_w on the lag phase duration and growth kinetics of *E. coli* and other foodborne bacteria are examined specifically in this thesis. The physiological history of the cell is thought to have an affect on lag phase duration, and this is examined for *S.* Typhimurium and a_w downshifts. The relationship between 'cell yield' and relative lag time and lag rate is investigated to determine whether the 'cell yield' response to a_w can be related to a normal physiological range for a_w. The relative lag time concept (RLT) is explored to test the hypothesis that lag times can be understood in terms of the *amount* of work to be done in adjusting to a new environment and the *rate* at which that work is done. Additionally, the reliability and reproducibility of RLT estimates is examined. The potential effect of choice of recovery medium, diluent and temperature of incubation on lag and generation time estimates for osmotically injured cells is also considered.

The significance of the results presented in this thesis for the meat industry is discussed.

2. GENERAL MATERIALS AND METHODS

2.1.1 Cultures, Equipment and General Procedures

Details of bacterial strains used are found in Appendix, A., 9.1.1. Equipment used is listed in A. 9.2. All basal media were prepared as per manufacturers' instructions. Details of media preparation and formulations are given in A. 9.3.1 and A. 9.3.2. The pH of media was determined post-autoclaving and adjusted aseptically by the addition of sterile 0.1M HCl or 0.1M NaOH if required. For NaCl modified media, the a_w of the media post-autoclaving was determined from triplicate measurements using a dew point a_w meter (A. 9.2).

To allow comparisons between experiments, procedures such as inoculum preparation and construction and analysis of growth curves were standardised.

2.1.2 Inoculum preparation

Unless otherwise stated, late exponential phase inoculum was used. The late exponential phase cultures, or secondary inocula, were prepared from a primary stationary phase broth culture as described in S. 2.1.2.1. The late exponential phase inocula were considered to contain a mix of predominantly exponential phase cells and some stationary phase cells. In later experiments examining the effect of the physiological history of the cell, purely exponential phase and stationary phase cultures were required. Their preparation is described in S. 6.2.1.1. All inocula were placed in ice-water baths immediately after preparation to minimise changes in culture density.

Prior to the commencement of each experiment, the appropriate isolate was resuscitated from cryogenic storage as described in A. 9.1.3.

2.1.2.1 Late exponential phase inoculum

A primary inoculum was prepared by taking a loopful of an appropriate 24 hour plate culture and inoculating it into 50mL of pre-warmed medium in a sterile 250mL stoppered conical flask. The broth was incubated in a

shaking water bath at 25 (±0.1)°C for a time sufficient to provide stationary phase cells. This incubation time was determined from previous knowledge of growth rate of each of the strains at 25°C. This was ~14 hours for *K. oxytoca, E. coli* and *S.* Typhimurium strains, ~20 hours for *L. monocytogenes* and ~26 hours for *S. aureus* . Unless stated otherwise, *S. aureus* and *E. coli* strains were generally prepared in Nutrient Broth (NB), *K. oxytoca* and *S.* Typhimurium in Brain Heart Infusion Broth (BHI) and *L. monocytogenes* in Tryptone Soya Broth with 0.6% Yeast Extract added (TSB-YE).

For the secondary inoculum, 50mL of liquid media in a 125mL side-arm flask was used. A second side-arm flask containing the same medium was used as a blank for spectrophotometric determinations. A small volume of the primary inoculum, typically 0.3mL, was aseptically dispensed into the medium and the flask incubated in a shaking water bath at 25 (± 0.1) °C. Growth was monitored turbidimetrically at 540nm with an analogue spectrophotometer. The culture was incubated until transmittance had dropped to 20%. At this density cells were in the late exponential phase of growth at a concentration of ~10^{7-8} cells.mL^{-1}. For viable count determinations a lower inoculum density was required. The inoculum was serially diluted in 0.1% Peptone Water, PW, unless otherwise stated, to a concentration of ~10^{3-4} cells.mL^{-1}.

The medium used for growth of the secondary inoculum was dependent on the type of experiment performed and is detailed in the subsequent sections. For temperature shift experiments the medium used was the same as that of the primary inoculum. For a_w shift experiments, some of the secondary inocula were cultured in NaCl modified broth.

2.1.3 Construction and analysis of growth curves

2.1.3.1 Construction of growth curves

2.1.3.1.1 Turbidimetric

Growth curves were constructed by measuring per cent transmittance (%T) at 540nm with a digital spectrophotometer. A small volume of inoculum was added to test broths to achieve a ~5% drop in transmittance.

The turbidity of the test broth was monitored, with measurement times chosen to correspond, approximately, to successive drops in transmittance of ≤5%. More recordings were taken around the inflection points of the growth curves, in particular the initial section of each curve, to increase confidence in determination of the end of the lag phase. Each tube or sidearm flask was monitored until %T ceased to change, assessed as three successive readings without change in %T. %T values were converted to OD values using Eqn. (6):

$$OD = 2\text{-}Log_{10}\%T \qquad \text{Eqn. (6)}$$

Log OD was plotted against time. Unless otherwise stated growth curves generated in complex laboratory media were analysed by fitting a modified Gompertz equation (S. 2.1.3.2) Growth curves constructed in minimal media, i.e. those for 'cell yield' experiments, were analysed by linear regression (S. 2.1.3.3).

2.1.3.1.2 Viable Count

A single experiment employed the use of PECC dry film plates for determinations of viable counts. The methodology employed for their use is detailed in S. 4.2.1.1.1. For all other viable count determinations a spread plate technique was used. At regular time intervals, 0.1 mL aliquots were removed from the test sample, serially diluted as required and surface plated onto duplicate sets of appropriate agar plates. Plates were incubated and colonies counted manually. Details of diluents, media and incubation times used for each experiment are given in the following sections.

Log_{10}(viable counts) were plotted against time and growth curves analysed by linear regression (S. 2.1.3.3).

2.1.3.2 Non-linear regression analysis

A number of mathematical functions have been proposed to describe the sigmoid growth curve characteristic of microbial growth in batch culture (McMeekin *et al.*, 1993). The modified Gompertz equation is widely used

and can be fitted to either Δ%T, log viable count or log optical density data generated in complex laboratory media, and log viable count data from food inoculation studies. The equation takes the form:

$$y(t) = A + D \exp\{-\exp[-B(t - M)]\} \qquad \text{Eqn. (7)}$$

where $y_{(t)}$= population density, Log N (cfu.mL^{-1}) or Log OD or Δ%T, at time *t*
t = time
A= population density at t = - ∞ [i.e. Log $N_{(-\infty)}$, Log $OD_{(-\infty)}$ or Log $\%T_{(-\infty)}$]
D= difference between final and initial population density [i.e. Log $N_{(\infty)}$ - Log $N_{(-\infty)}$, Log $OD_{(\infty)}$ - Log $OD_{(-\infty)}$ or $\Delta\%T_{(\infty)}$-$\Delta\%T_{(-\infty)}$]
B= is related to the relative growth rate at M
M= time at which the exponential growth rate is maximal

The modified Gompertz equation is non-linear, and was fitted by non-linear regression using the SAS PROC.NLIN routine (SAS Institute Inc., Cary, N.C.) to estimate the above parameters for each data set. By differentiating Eqn. (7) with respect to time, the lag phase duration and generation time for viable count, optical density or Δ%T data can be defined in terms of the equations' parameters. Derivation of the resulting equations can be found in (McMeekin *et al.*, 1993):

$$GT \text{ (viable count or log OD)} = \frac{0.81828}{BD} \qquad \text{Eqn. (8)}$$

$$GT (\Delta\%T) = \frac{66.59}{BD} \qquad \text{Eqn. (9)}$$

$$Lag = M - \left(\frac{1}{B}\right)[1 - \exp(1 - \exp(BM))] \qquad \text{Eqn. (10)}$$

where the terms are as defined previously.

In general, the modified Gompertz function over-predicts the slope of the growth curve and some workers have chosen to apply a correction factor, i.e. multiplication by 1.13 (*see* S. 3.1.3), to the above equations which then take the form:

$$GT \text{ (viable count or log OD)} = 1.13 \left(\frac{0.81828}{BD}\right) \quad \text{Eqn. (11)}$$

$$GT (\%T) = 1.13 \left(\frac{66.59}{BD}\right) \quad \text{Eqn. (12)}$$

$$Lag = M - \frac{1.13}{B}\left[1-\exp(1-\exp(BM))\right] \quad \text{Eqn. (13)}$$

Negative lag time estimates can occur (McMeekin *et al.*, 1993), but are usually avoided by use of Eqn. (13) (Ross, 1993). However problems can be encountered for aberrant growth curves such as those with no initial period during which the population density was static, or an ill defined or gradual increase towards the stationary phase. In this thesis it is proposed to interpret negative lag time estimates generated by Eqn. (13) as a lag time of zero for calculation of relative lag times.

2.1.3.3 Linear regression analysis

Linear regression analysis was used when growth curves did not conform to the characteristic sigmoid shape required for analysis by the modified Gompertz equation. Krist *et al.* (1998a) observed that growth curves generated by turbidimetric data in glucose-limited minimal media demonstrated a sudden cessation of optical density increase at substrate exhaustion. Growth rates estimated from fitted Gompertz equations were not appropriate. In that situation, linear regression provided a better estimate of growth rate. Unless otherwise stated, turbidimetric data generated in complex laboratory and minimal media were analysed by this method. Linear regression analysis was also used in this thesis for viable count data as fewer data points were generally collected at the inflection points of the growth curve and under stressful conditions the early stages of the growth curve may be altered due to injury (*see* S. 4.1.1.4.1). A representative turbidimetric data set is plotted in Fig. 2.1.

Linear regression was used to calculate generation time and lag time as follows. Generation time was defined as the time for the bacterial population to double in cell numbers. A straight line was fitted to a visually selected set of points that appeared to represent the exponential phase of growth using the program Cricket Graph v.1.3.2 (Cricket

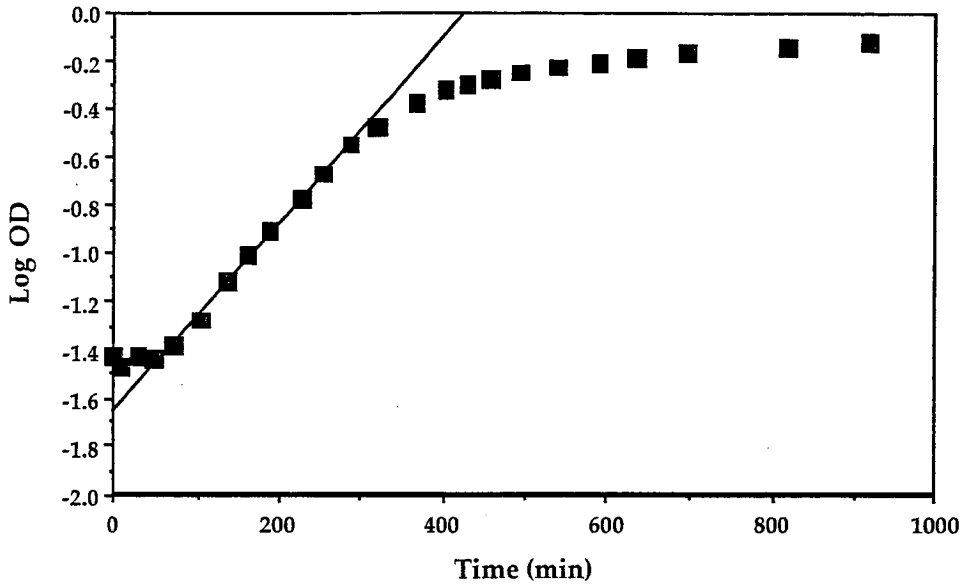

Figure. 2.1: Growth of *E. coli* SB1 in 1/4 strength Nutrient Minimal Broth, at a_w 0.979 and 25.3 (±0.2)°C.

Software, Malvern, PA 19355). Typically this region included ≥6 to 15 points. The regression line is of the form:

$$y = c + mx \qquad \text{Eqn. (14)}$$

where

y	=	log numbers
c	=	the *y* axis intercept
m	=	the slope
x	=	time

The generation time is calculated by dividing the slope, *m*, by 0.301 (equivalent to $\log_{10}2$), thus:

$$GT = \frac{0.301}{m} \qquad \text{Eqn. (15)}$$

For example, in Fig. 2.1 the regression line is described by the equation:

$$y = -1.6599 + 3.8914e\text{-}3x \qquad \text{Eqn. (16)}$$

Substituting *m* from Eqn. (16) the generation time for the growth curve in Fig. 2.1 is:

$$GT = \frac{0.301}{3.8914e\text{-}3} = 77.4 \text{ minutes}$$

Various definitions of lag are found in the literature, and the definition employed depends on the mathematical model or curve fitting procedure applied to the growth data (Buchanan and Cygnarowicz, 1990; Zwietering *et al.*, 1991; Zwietering *et al.*, 1992). In this thesis lag time is depicted by the value on the *x* axis where the intercept of the regression line through the exponential part of the growth curve equals the starting *y* value. Lag time may be calculated by:

$$Lag = \frac{y_{(initial)} - c}{m} \qquad \text{Eqn. (17)}$$

For example, using Eqn. (17) and substituting values for *c* and *m* from Eqn. (16) and reading '$y_{(initial)}$' from Fig. 2.1:

$$Lag = \frac{-1.4300 + 1.6599}{3.8914e\text{-}3} = 59.09 \text{ minutes}$$

2.1.4 Preparation of NaCl modified media

For *K. oxytoca* model development (Chapter 3) and osmotic shift experiments (Chapter 5) a series of broths covering the growth permissive a_w range for each species was required. Information on a_w minima was obtained from Jay (1997) for *Salmonella*, Troller and Christian (1978) for *E. coli*, Ash (1997) for *S. aureus*, Ørskov (1984) for *K. oxytoca* and Tienungoon (1999) for *L. monocytogenes*.

Basal laboratory media and basal media with added NaCl were prepared. Sufficient NaCl was added to the appropriate basal laboratory medium to provide a broth with a a_w near the minimum for growth. The amount of NaCl required to provide broths of these a_w was determined from the tables of Chirife and Resnik (1984). The pairs of basal media (in which the a_w was measured) were combined in various ratios to generate a series of

broths at a range of a_w values. The number of ratios tested is detailed in the appropriate materials and methods section of each chapter. 15mL aliquots of the a_w adjusted media were dispensed into sterile L-tubes.

For each L-tube, the salt concentration was calculated from the ratios of each medium mixed, and the a_w determined from the tables of Chirife and Resnik (1984). The final a_w of broth in each L-tube for a_w shift experiments was calculated by including the effect of the a_w and volume of the inoculum to be added. A subsample of inoculated broths was measured and compared to the calculated a_w values. In each case the measured value was within the measurement limits of the a_w meter, i.e. ±0.003 of the calculated value.

The a_w of broths used for growth of primary and secondary inocula, and the broths used for preparation of the test broths by mixing, were measured by triplicate readings using a a_w meter. The measured a_w is referred to throughout the thesis. Where NaCl was added to modify the a_w of broths, the amount was also recorded.

Unless otherwise stated, the calculated, theoretical a_w values for *test broths* dispensed in L-tubes are used throughout the remainder of this thesis. This method of reporting a_w was used to obviate the time consuming process of triplicate a_w measurements on large numbers of broths. Additionally, measurement of the a_w immediately post-inoculation, i.e. to determine changes in a_w due to introduction of an inoculum at a different a_w to that of the test broth, would necessitate removal of aliquots from the inoculated test broths, a process which may introduce unwanted contaminants.

Prior to inoculation, L-tubes containing the a_w adjusted media were equilibrated for 12 hours with shaking (32-40 oscillations.min^{-1}) on a temperature gradient incubator (TGI) set to operate at the temperature required for the experiment. The temperature, and variation in temperature, of each position in the incubator was determined from triplicate measurements with a digital thermometer in each L-tube upon completion of the experiment. For osmotic shift experiments the TGI's

were set isothermally at 25.5 (± 0.4)°C. For temperature shift experiments, the TGI was set to provide conditions for the growth permissive range. Information on temperature maximum and minima were obtained as for a_w.

3. MODEL DEVELOPMENT

3.1 INTRODUCTION

An absolute prerequisite to the development of reliable mathematical models is the collection and appropriate processing of good quality data describing the effects of different factors on microbial development McMeekin *et al.* (1993). This chapter describes the development of two kinetic models for microbial growth and employs the basic concepts and methods described by McMeekin *et al.* (1993) to produce reliable predictive models.

3.1.1 A square-root type model for *E. coli* growth

The long history of use of *E. coli* as an experimental organism, primarily as an indicator organism and latterly as a serious foodborne pathogen, is reflected in the amount of data available describing its growth. There are many published kinetic models for *E. coli* growth (Buchanan *et al.*, 1993; Buchanan and Bagi, 1994; Gill and Phillips, 1990; Heitzer *et al.*, 1991; Kovárová *et al.*, 1996; Rosso *et al.*, 1995; Sutherland *et al.*, 1995; Presser *et al.*, 1997). These models are constructed for a variety of strains, substrate and environmental parameters (both constant and varying). Although there are a number of growth models for *E. coli* in the literature or commercially available, it is preferable to use a model specific to the type of food or process.

Previous models for *E. coli* growth are limited to modelling the effects of pH, for example Rosso *et al.* (1995), and not the acidulant. The square-root type model of Presser *et al.* (1997), Eqn. (5) presented in S. 1.5.1.1, has five terms describing the inhibition of *E. coli* growth due to temperature, a_w, pH, the dissociated form of lactic acid and the undissociated form of lactic acid. Eqn. (5) was extended to include terms for the super-optimal pH and temperature ranges. The model takes the form:

$$\sqrt{k} = (c(T-T_{min})(1-\exp(d(T-T_{max})))\sqrt{(a_w-a_{wmin})}\sqrt{1-10^{(pHmin-pH)}})\sqrt{(1-10^{(pH-pHmax)})}\sqrt{1-[LAC]/U_{min}}(1+10^{(pH-PKa)})\sqrt{1-[LAC]/D_{min}}(1+10^{(pKa-pH)})\pm e) \quad \text{Eqn. (18)}$$

where: k = specific growth rate, c = fitted parameter, T = temperature (°C), T_{min} = theoretical minimum temperature for growth, d = fitted parameter, T_{max} = theoretical maximum temperature for growth, a_w = water activity, $a_{w\,min}$ = theoretical minimum a_w for growth, pH_{min} = theoretical minimum pH for growth, pH_{max} = theoretical maximum pH for growth, [LAC] = lactic acid concentration (mM), U_{min} = minimum concentration of undissociated lactic acid which prevents growth, D_{min} = maximum concentration of dissociated lactic acid which prevents growth, pKa = pH at which levels of undissociated and dissociated lactic acid are in equilibrium and e = error term.

It should be noted that although the effects of super-optimal a_w can be included in square-root type models, *see* S. 1.5.1.1 - Eqn. (4), the $(1-\exp\{d(a_w-a_{wmax})\})$ term was not included in Eqn. (18). This term could not be easily estimated as there were insufficient data at a_w >0.997 as all experiments were undertaken in full-strength laboratory media.

3.1.2 A predictive model for use in industry trials

Performance evaluation of a predictive model in product subjected to the normal processes of production, distribution and storage is the most rigorous test of model efficiency. However, the deliberate introduction of a pathogen such as *E. coli* to the processing environment is precluded. The use of a surrogate organism, *K. oxytoca* was proposed and a square-root type model for growth of *K. oxytoca* was required for use in carcass chilling verification studies.

A simplified form of the kinetic square-root type model introduced by Presser *et al.* (1997) was proposed:

$$\sqrt{k} = (b((T-T_{min})(1-\exp(c(T-T_{max})))\sqrt{(a_w-a_{wmin})}(1-\exp(d(a_w-1)))1-\frac{10^{pHmin}}{10^{pH}}))\pm e$$

Eqn. (19)

where: k = maximum specific growth rate, b, c and d= fitted parameters, T = temperature (°C), T_{min} = theoretical minimum temperature for growth, T_{max} = theoretical maximum temperature for growth, a_w = water activity, $a_{w\,min}$ = theoretical minimum a_w for growth, pH_{min} = theoretical minimum pH for growth and e = error term.

Unlike Eqn. (18), terms for super-optimal pH and the dissociated and undissociated forms of lactic acid were not included. As the model was intended for use in a carcass chilling trial, pH values likely to be encountered would fall in the neutral to acidic range. However, it should be noted that some of the growth data was generated in broths to which lactic acid was added to simulate pH and [LAC] conditions based on the values of Grau (1981) for beef. Thus, although terms for lactic acid were not specifically included in the model, some data was collected under conditions likely to be encountered in the food ecosystem.

Some of the growth rate data included in the growth model for *K. oxytoca* was obtained from experiments which examined the effects of a_w and temperature shifts on lag phase duration of exponentially growing cells of *K. oxytoca* (*see* S. 5.2.1.1 and S. 5.2.2.2). Data to compare turbidimetric data to viable count observations was collected to investigate the need for a correction factor to be applied to the model.

3.1.3 Correction factors

When comparing the data of other researchers to a model it is important to consider differences in experimental techniques and analytical methods. Two areas have been identified which may require a correction factor to be applied: comparing turbidimetric data to viable count data and using a curve fitting function which may over-predict growth rate.

The modified Gompertz function was introduced by Gibson *et al.* (1987) after modification of the model given by Gompertz in 1825. It fits a non-symmetrical sigmoid curve to either optical density, viable count or Δ%T data. However, the curve generated by the modified Gompertz function does not fit a straight line through the exponential phase of growth. It has

a definite curvature around an inflexion point, and overestimates maximum specific growth rate (Baranyi *et al.*, 1993b). Analysis of the data presented in Whiting and Cygnarowic-Provost (1992) revealed an overestimation averaging 21 (±17)%. Similarly for data in Baranyi *et al.* (1993b) the overestimation averages 17 (±20)%. These analyses are based on experimental growth curve data covering a range of conditions at various levels. Ross (1993) undertook a systematic examination using idealised growth curves to reduce the effects of variation inherent in experimental data. His analyses determined the overestimation of the true slope to lie between 11 and 15%. Similarly Dalgaard *et al.* (1994) found the modified Gompertz function to overestimate by 12 (± 5)%. Consequently, researchers at the University of Tasmania routinely employ a correction factor of 13%, deemed a reasonable average, to growth rate estimates derived from the modified Gompertz function.

The lower sensitivity limit of turbidity measuring devices is such that, in general, they are unable to detect populations of less than about 10^7 cfu.mL^{-1} (Dalgaard *et al.*, 1994). Consequently, turbidimetric measurements change only during a narrow region of the growth curve and this region may not be representative of the population at maximum exponential growth. When comparing turbidimetric estimates of growth rate with the more sensitive method of viable counts, Dalgaard *et al.* (1994) observed a systematic deviation between the two. Therefore turbidimetric estimates of growth rates may be equated with viable count estimates by multiplication with a calibration factor. Growth rates estimated from Δ%T data generated by the modified Gompertz equation should be multiplied by 1.6 to equate to viable count data. Growth rate estimates from absorbance data should be multiplied by a factor of 1.1 (Dalgaard *et al.*, 1994).

As much of the literature for *E. coli* presents kinetic data determined from viable count assays it is important to consider the application of correction factors for successful comparison to data generated by Eqn. (18). Generation time estimates using Eqn. (18) have been modified to include the correction factor 1.6 as Δ%T data was used in the model construction.

The curve fitting program used in this study is based on the modified Gompertz function, thus the correction factor of 1.13 is also applied.

3.1.4 Objectives

In this chapter a new model is proposed and developed to describe specifically the growth of *E. coli* on meat under Australian commercial practice. A second model for another gram-negative organism, *K. oxytoca* is also proposed and developed.

3.2 MATERIALS AND METHODS

3.2.1 Collection of growth data for *E. coli*

Turbidimetric growth rate data (%T) was collated from Presser *et al.* (1997), Salter (1998) and novel data collected by the candidate (refer to S. 5.2.1) for *E. coli* SB1 and *E. coli* R31. The raw data are presented in A. 9.4.1. The modified Gompertz function was fitted to % transmittance data according to the method of McMeekin *et al.* (1993).

3.2.2 Collection of growth data for *K. oxytoca*

Growth rate data for *K. oxytoca* was collected for the temperature range 5 to 45°C, the a_w range 0.943 to 0.996 and for pH (lactic acid) conditions relevant to meat (pH 5.5 in the presence of 130 mM lactic acid and pH 6.1 in the presence of 85 mM lactic acid (Grau, 1981).

Some of the growth rate data included in the growth model for *K. oxytoca* was obtained from experiments which examined the effects of a_w and temperature shifts on lag phase duration of exponentially growing cells of *K. oxytoca* (*see* S. 5.2.1.1 and S. 5.2.2.2).

Data to compare turbidimetric data to viable count observations was collected to investigate the need to apply a correction factor (S. 3.1.3) to the model. An experiment designed specifically to test eight different temperature, a_w, pH and lactic acid concentration combinations was undertaken. An additional set of analogous optical density and viable

count generation time estimates were obtained from an experiment which examined the effect of abrupt osmotic shifts on the RLT of *K. oxytoca* in BHI at pH 7.20 (*see* S. 5.2.1.4). It must be noted that due to the dilute inoculum, the data collected turbidimetrically does not represent the true population lag because the initial inoculum was not detectable turbidimetrically until some growth had occurred. However, data collected for the growth curves is valid for calculation of generation time using non-linear regression.

3.2.2.1 pH and lactic acid adjusted broths at various temperatures

OVERVIEW

A primary inoculum in stationary phase was prepared in basal BHI. Aliquots were removed and inoculated into pH and lactic acid modified BHI and incubated to provide a secondary inoculum of late exponential phase culture. The secondary inoculum was dispensed into pH and lactic acid modified broths at 30 temperatures in the range 0.5 to 47.0°C. Growth was monitored turbidimetrically and growth curves analysed using non-linear regression. Cardinal values for temperature were obtained.

METHOD

A late exponential phase inoculum of *K. oxytoca* in BHI was prepared according to S. 2.1.2.1. Two pH and lactic acid modified BHI broths were used to grow the secondary inocula of late exponential phase cultures: BHI with 85mM lactic acid at pH 5.55, BHI-85, and BHI with 130mM lactic acid at pH 6.1, BHI-130.

The two media types, i.e. 15 of each, were incubated in alternating positions along one side of a temperature gradient incubator, TGI-1. The temperature range was set for 0.5 to 47.0°C.

0.3mL of the appropriate exponential phase inoculum was aseptically dispensed into each of the 15 L-tubes of the appropriate pH and lactic acid modified BHI broths.

Growth was monitored turbidimetrically according to S. 2.1.3.1.1. Generation time and lag times were estimated using non-linear regression (S. 2.1.3.2).

3.2.2.2 pH and lactic acid adjusted broths at various a_w

OVERVIEW

A primary inoculum in stationary phase was prepared in basal BHI. Aliquots were removed and inoculated into pH and lactic acid modified BHI and incubated to provide a secondary inoculum of late exponential phase culture. The secondary inoculum was dispensed into pH and lactic acid modified broths at various a_w covering the range a_w 0.943 to a_w 0.988. Growth at 25.5 (± 0.4)°C was monitored turbidimetrically and growth curves analysed using non-linear regression.

METHOD

A late exponential phase inoculum of *K. oxytoca* in BHI was prepared according to S. 2.1.2.1. Two pH and lactic acid modified BHI broths were used to grow the secondary inocula of late exponential phase culture: BHI-85 and BHI-130.

15mL broths covering a range of a_w were prepared by mixing BHI-85 (a_w 0.988) and BHI-130 (a_w 0.991) with BHI-85 with 80g.L^{-1} NaCl added (a_w 0.944), BHI-85-S, and BHI-130 with 80g.L^{-1} NaCl added (a_w 0.943), BHI-130-S, respectively in ratios as described in S. 2.1.4. 20 broths for each pH/lactic acid combination were prepared.

A temperature gradient incubator, TGI-1, was set to operate isothermally at 25.5 (± 0.4)°C. Each side of TGI-1 contained 24 ports, and each media type occupied one side.

0.3mL of the appropriate late exponential phase inoculum was aseptically dispensed into each of the 24 L-tubes of the appropriate pH/ lactic acid and a_w modified BHI broths.

Growth was monitored turbidimetrically according to S. 2.1.3.1.1. Generation time and lag times were estimated using non-linear regression (S. 2.1.3.3).

3.2.2.2.1 Viable count and optical density calibration

OVERVIEW

To account for possible differences between growth rates observed by turbidimetric and viable count data, a late exponential phase inoculum in basal BHI was dispensed into a variety of sterile liquid media and growth at various temperatures monitored by viable count and turbidimetry. The test broths were either basal, NaCl or pH/lactic acid modified sterile liquid media and incubation temperatures are detailed in Table 3.1. Growth rate estimates from both techniques were compared.

Table 3.1: Broths used to monitor the growth of *K. oxytoca* by optical density and viable count

Basal Medium	Temperature (°C)	a_w	pH	Lactic acid (mM)	Container
BHI	40.0	0.996	7.30	0	sidearm flask
BHI	33.6	0.996	7.30	0	L-tube
NB	25.3	0.952	7.30	0	L-tube
NB	25.3	0.990	7.30	0	L-tube
BHI	37.0	0.993	6.10	85	sidearm flask
BHI	19.9	0.950	6.10	85	L-tube
BHI	9.9	0.988	5.55	130	L-tube

An additional set of analogous optical density and viable count generation time data was obtained from an experiment which examined the effect of abrupt osmotic shifts on the RLT of *K. oxytoca* in BHI at pH 7.20 (*see* S. 5.2.1.1).

METHOD

A late exponential phase inoculum of *K. oxytoca* in either BHI or Nutrient broth, NB, was prepared according to S. 2.1.2.1. The secondary inoculum was serially diluted in PW, to a concentration of ~$10^{5\text{-}6}$ cfu.mL^{-1}. Prior to inoculation into each test broth, the viable count of the diluted inoculum was determined by spread plating 0.1mL aliquots of appropriate dilutions on Plate Count Agar (PCA) and incubating plates at 35°C for 18 to 24

hours. Colonies were counted manually and the concentration of the cells in the diluted inoculum was estimated. This estimate was used to calculate the concentration of cells in each of the test broths after addition of the diluted inoculum, i.e. the additional dilution upon inoculation into the broth is taken account of. These estimates were plotted as the viable count at time zero.

The preparation of the basal, NaCl or pH/lactic acid modified sterile liquid media was described previously. 15mL aliquots of the appropriate media were dispensed into L-tubes and 50mL of broth were dispensed into 125mL sidearm flasks. Side-arm flasks were pre-warmed in shaking waterbaths at the appropriate temperature and L-tubes were pre-warmed with shaking in TGI-2 set isothermally at 25.5 (± 0.4)°C.

0.5mL of undiluted inoculum was dispensed into L-tubes and growth monitored turbidimetrically (S. 2.1.3.1.1). 1.5mL of diluted inoculum was added to side-arm flasks and growth monitored by viable count on PCA.

For the viable count data, lag and generation times were estimated using linear regression (S. 2.1.3.3). Relative lag times and lag rate were calculated.

3.3 RESULTS

3.3.1 A square-root type model for *E. coli*

The growth rate model for *E. coli* (Eqn. 18) was fitted to the data by Dr D Ratkowsky (University of Tasmania) using the SAS non-linear regression routine PROC.NLIN (SAS Institute Inc., Cary, N.C.) and generated parameter estimates shown in Table 3.2.

Table 3.2: Parameter values for the growth rate model for *E. coli* .

Parameter	Estimate	Asymptotic Standard Error
c	0.2345	0.0083
T_{min}	4.14	0.63
T_{max}	49.55	0.42
pH_{min}	3.909	0.031
pH_{max}	8.860	0.186
U_{min}	10.433	0.521
D_{min}	995.509	106.372
$a_{w\,min}$	0.9508	0.0004
d	0.2636	0.0375
e (RMSE)*	0.0054	
		*RMSE=Root Mean Square Error

3.3.2 A square-root type model for *K. oxytoca*

The generation time data collected from experiments detailed in S. 3.2.1.2.1, S 3.2.1.2.2, S. 5.2.1.1 and S. 5.2.2.2, were fitted to Eqn. (19) using a SAS PROC.NLIN routine. The generation time data modelled are presented in A. 9.4.2, A. 9.4.3, A. 9.4.12 and A. 9.4.19.

The following parameter estimates were generated (Table 3.3).

Table 3.3: Parameter values for a square-root type model for *K. oxytoca* (Eqn. 19)

Parameter	Estimate	Asymptotic Standard Error
b	0.0317	0.0018
T_{min}	0.49	1.27
c	0.3403	0.0538
T_{max}	47.18	0.22
$a_{w\,min}$	0.9462	0.0007
pH_{min}	4.747	0.175
e (RMSE)	0.0141	

As evidenced by the low RMSE, Eqn. (19) describes the data well.

3.3.3 Correction factors

3.3.3.1 Eqn (18): *E. coli*

As recommended by Dalgaard *et al.* (1994), a correction factor of 1.6 is used for comparisons to viable count data, and 1.1 for absorbance data.

3.3.3.2 Eqn (19): *K. oxytoca*

Eqn. (19) was generated from turbidimetric data therefore a correction factor for comparisons to viable count data was required (Table 3.4). The correction factor for comparison of optical density to viable count data was estimated to be 1.28 (±0.30), i.e. generation time estimates from optical density data should be divided by 1.28 when comparing to viable count data.

Table 3.4: Comparison of generation time estimates from analogous viable count and optical density determinations under various growth conditions

Medium	°C	a_w	pH	[LAC]	Vessel	GT determined from: VC	OD	OD:VC
Direct comparison (VC compared with OD estimate)								
BHI	40.0	0.996	7.30	0	SA	19.9	25.1	1.26
BHI	33.6	0.996	7.30	0	LT	23.4	20.8	0.89
NB	25.3	0.952	7.30	0	LT	304.5	380.9	1.25
NB	25.3	0.990	7.30	0	LT	40.8	45.3	1.11
BHI	37.0	0.993	6.10	85	SA	21.5	22.9	1.06
BHI	19.9	0.950	6.10	85	LT	626.9	628.3	1.00
BHI	9.9	0.988	5.55	130	LT	538.6	968.4	1.80
							Average	**1.17**
							Standard Error	**± 0.30**
Indirect comparison (RLT experiment-see S. 5.2.1.3)								
BHI	25.3	0.992	7.20	0	LT	43.33	40.88	0.94
BHI	25.3	0.984	7.20	0	LT	42.25	55.13	1.30
BHI	25.3	0.976	7.20	0	LT	46.25	74.62	1.61
BHI	25.3	0.968	7.20	0	LT	54.56	97.80	1.79
BHI	25.3	0.960	7.20	0	LT	84.44	130.02	1.54
BHI	25.3	0.953	7.20	0	LT	192.76	227.86	1.18
BHI	25.3	0.949	7.20	0	LT	270.59	346.62	1.28
BHI	25.3	0.945	7.20	0	LT	459.49	737.14	1.60
							Average	**1.38**
							Standard Error	**± 0.28**
							Overall Average	**1.28**
							Overall Standard Error	**± 0.30**

BHI=Brain Heart Infusion Broth, NB=Nutrient Broth, SA=Sidearm flask, LT=L-tube, VC=Viable Count and OD=Optical Density

3.4 DISCUSSION

The square-root type model of Presser *et al.* (1997), Eqn. (5) presented in S. 1.5.1.1, has five terms describing the inhibition of *E. coli* growth due to

temperature, a_w, pH, the dissociated form of lactic acid and the undissociated form of lactic acid. This equation was developed for application to foods in which the pH was generally in the range of acidic to neutral and in which temperatures were suboptimal. Carcass meats generally fall into these categories. However, in the interest of obtaining a more complete, and thus more versatile model suitable for application to a wider range of foods, Eqn. (5) was extended to include terms for the super-optimal pH and temperature ranges, resulting in a new square-root type model for *E. coli* growth, Eqn. (18). The absence of a correction factor for Eqn. (18) has been noted and it is recommended that those described in Dalgaard *et al.* (1994) be used if the model is to be compared to other data.

The model for *K. oxytoca* growth rate, Eqn. (19), encompasses the effects of temperature, a_w, and pH/lactic acid conditions of relevance to meat. As the model was produced from turbidimetric data it was necessary to calculate a calibration factor to equate the model estimates of growth rates to viable count estimates. The correction factor was estimated to be 1.28 (Table 3.4). This estimate is similar to, although a little higher, than that determined by Dalgaard *et al.* (1994) who suggested that growth rate estimates from absorbance data should be multiplied by a factor of 1.1 to make them equivalent to growth rate determinations performed by total aerobic counts.

4. MODEL VALIDATION

4.1 INTRODUCTION

4.1.1 Model performance and evaluation

Kinetic models are generally based on data for microbial growth in conventional laboratory broths. Liquid media are homogeneous, allow uniform distribution of organisms, are easily modified and suitable for a range of detection methods. However liquid media in most cases do not represent the food matrix. Generation of data for kinetic models using food as the growth substrate presents a number of problems, mainly in application of the test organism, lack of homogeneity (unless using a liquid food such as milk), presence of normal spoilage biota as well as logistical problems with enumeration techniques. Practicality dictates that in most situations the model be generated in laboratory media.

Prior to application in industry, predictive models require some performance assessment under novel conditions that were not used to derive the models and they "must be shown to predict accurately the behaviour of microorganisms in foods during processing, storage and distribution" (Ross, 1996). This process is termed validation and is "a useful and necessary part of the modelling procedure" (McMeekin *et al.*, 1993). There are several ways in which model performance may be assessed. These include using sub-sets of the data set from which the model is derived, generating new data by either laboratory experiments in liquid growth media or direct inoculation into product, comparison to other data in the literature and trials in industry.

It is interesting to note the interpretation of the term 'validation' in the literature by authors. The term evaluation, i.e. to assess, is often more appropriate than validation, i.e. to make valid or confirm, as many predictive models are never investigated in the production environment. Neumeyer *et al.* (1997) correctly refer to their work on pseudomonad growth as a validation; industry trials were performed in addition to laboratory and literature performance evaluations. Dalgaard and Jørgensen (1998) correctly refer to their seafood challenge tests as

evaluations. Similarly Augustin and Carlier (2000), refer to their literature assessment of a *L. monocytogenes* model as a performance evaluation. Despite not applying objective criteria, Walls and Scott (1996) entitled their work on challenge tests in raw ground beef as a validation.

Some techniques for assessing model performance are described below. Bearing in mind the previous discussion, if the techniques are used in isolation then they constitute evaluations only. However, it is proposed that if the techniques are used collectively, then the term 'validation' is appropriate.

4.1.1.1 Laboratory performance evaluation

Laboratory based performance evaluation studies may be conducted in various laboratory media or performed as challenge tests where the target organism is directly inoculated into or onto the product. The microbial growth or survival is then enumerated.

Performance evaluations in complex laboratory media are arguably the easiest to undertake for the same reasons that models are constructed in liquid media. In contrast, "the physiochemical environment on and in foods is spatially and temporarily heterogeneous and extremely complex" (Wimpenny *et al.*, 1995). Liquid media in most cases do not represent the food matrix, therefore predictive models require performance evaluation in the food itself, "preferably under fluctuating temperature regimes that simulate those likely to be encountered in commercial practice" (McMeekin *et al.*, 1993). However, other environmental factors affect microbial growth; pH and a_w are discussed in S. 1.2. Additional factors which may require investigation include atmospheric conditions, fat content, preservatives and initial microbial loads. The most practical approach is to examine factors which are easily measurable and of direct relevance to the nature of the food and the conditions it is likely to encounter from the place of manufacture through to its final destination with the consumer.

4.1.1.2 Literature performance evaluation

A complete performance evaluation of a laboratory generated model encompassing all combinations of factors affecting microbial growth can be considered either an enormous undertaking or an impractical one. The volume of work involved to test all combinations is immense, even if it entailed the examination of one or two of the primary variables of pH, temperature and a_w alone. Extending the performance evaluation process to food matrices increases the volume of work further, as well as providing potential complications in adjusting such variables in non-homogeneous matrices. An approach is required that allows an efficient examination of many variables with reasonable demands on time and resources.

Despite the apparent enormity of the task, model performance evaluation can proceed at a much more manageable level. Performance evaluation of an experimentally derived model by fitting data sets from the literature is logistically approachable as well as allowing researchers to identify "gaps" in the data. The direction and/or necessity of further performance evaluation experiments may then be more easily decided. Summaries of the literature growth data for various strains of *E. coli* and assessments of the performance of models against this data have been published (Buchanan *et al.*, 1993; Sutherland *et al.*, 1995).

When using data from the literature, those data should be assessed for its reliability. Protocols for acceptance or rejection of data must be established because publications do not always provide adequate information regarding experimental design, methods and food specifications. Consequently some assumptions regarding parameters such as pH and a_w may be required. These assumptions can be made quite reasonably, however, if other literature, particularly regarding food and media specifications, are utilised.

4.1.1.3 Industry performance evaluation

Ideally the ability of the model to predict accurately the behaviour of microorganisms in the food matrix itself would be assessed by

performance evaluation in the product. This usually involves direct inoculation of the target organism into or onto the product and tracing the growth or survival of the microorganism. Such a product performance evaluation can be conducted in the laboratory as previously discussed. However, the most rigorous test of model efficiency involves performance evaluation in product subjected to the normal processes of production, distribution and storage. This is termed industry performance evaluation.

Industry performance evaluation by deliberate application of bacteria to the product allows assessment of the model in 'real life' situations. The production environment may introduce factors affecting microbial growth which are not evident under laboratory conditions. For example, it has been reported that in seafood the lag phases of many microorganisms are short or absent (Dalgaard and Jørgensen, 1998). Such behaviour in a 'real life' situation is an important consideration for predictive models for all types of foods in which microbial proliferation may occur. Therefore, it is apparent that difficulties may arise in incorporating laboratory derived information into predictive models. Industry performance evaluation offers a valuable opportunity to collect useful information on lag phase duration. Incorporation of this information into models improves their predictive capability.

4.1.1.3.1 Carcass chilling trials

Industry performance evaluation allows the behaviour of the organism during processing, storage and distribution to be monitored. Industry performance evaluation, however, poses problems as a controlled and deliberate introduction of a pathogen such as *E. coli* into a processing environment is certainly not desirable, and in many circumstances precluded due to legislation. The value of industry trials as a performance evaluation technique, as well as providing important 'real-life' information on the lag phase, has been emphasised. Of particular relevance to the Australian meat industry is the effect of blast chilling on the microbiota of the carcass surface, in particular the effect on *E. coli*. Ideally, in order to assess the effectiveness of carcass blast chilling or other chill procedures *E. coli* would be applied to the carcass surface and monitored throughout an

abattoir. However, as mentioned above, this is not possible. Therefore, an alternative strategy is required.

K. oxytoca is a non-pathogenic, gram-negative enteric organism (Ørskov, 1984), similar in physiology to *E. coli*. In 1997 AQIS approved the use of this organism in a verification study assessing a hot water decontamination system at an abattoir in Oakey, Queensland (Ross, 1999). Researchers at Australian Meat Technology (AMT) in Brisbane, Australia, proposed the use of *K. oxytoca* in carcass chilling verification trials to act as a proxy for *E. coli*. Preliminary investigations conducted by CSIRO indicated that *K. oxytoca* shared similar growth characteristics to *E. coli* at temperatures between 10 and 37°C. AQIS subsequently agreed to use of *K. oxytoca* for a chilling verification study (I. Eustace, *pers. comm*, 1998).

Although similar rates of growth were observed for the two organisms, significant differences were noted near the temperature growth limits in the CSIRO study. Consequently a prerequisite for use of *K. oxytoca* as a direct indicator of behaviour of *E. coli* on beef carcasses was an assessment of its growth rates and limits under conditions encountered during carcass chilling, i.e. low temperature and a_w. A logical extension of this process was summarising the data in the form of a predictive model.

4.1.1.4 Methodological considerations

Performance evaluation of predictive models to be employed in the food industry poses many potential problems. The difficulties of constructing models in foods has already been highlighted and these same problems are encountered in food-based laboratory performance evaluations. Of particular concern are the presence of normal spoilage biota and potentially injured cells. Methods which alleviate such problems are needed.

Successful model evaluations in food may require manipulation of the food matrix to enable enumeration of target organisms, revision and alteration of growth media to allow growth of specific organisms which may or may not be injured, and modification of bacterial strains.

4.1.1.4.1 Bacterial injury

Microbial injury, repair and revival are important considerations in the food industry. Bacterial populations in food are often deliberately exposed to lethal treatments. If the treatment is not severe enough then the surviving bacteria are said to be injured or stressed. Treatments which are potentially injurious to bacteria include: heat, cold, drying, freeze-drying, freezing, cold shock, osmotic activity, irradiation, chemical treatments, starvation, low pH, food preservatives, and disinfectants (Hurst, 1977). Sublethally stressed cells retain their pathogenic traits (Mossel and van Netten, 1984), and are therefore of concern.

The expression of injury can take many forms but in essence it is the loss of ability of living cells to form visible colonies under some conditions (Hurst, 1977). Sublethal injury may be evidenced by the inability of bacteria to form colonies on common selective media that would otherwise support growth of cells not subjected to environmental stresses (Semanchek and Golden, 1998). This has significance for quantitative assessment of microbial populations.

Scheusner *et al.* (1971) observed that if the medium is highly selective then counts remain lower than those on a non-selective medium until growth approaches stationary phase. Results from Mackey and Derrick (1982b), for heat injured *S.* Typhimurium recovery on Tryptone Soya Agar in comparison to the same medium with 3% added NaCl, support his observation. Whilst injury is most evident on selective media, a similar phenomenon can be observed on non-selective media if the injury is severe enough.

Bacterial injury and estimating lag phase duration

Following a sufficiently injurious treatment, the number of colony forming units on selective media rapidly drops below the initial inoculum level and is followed by an increase in recoverability on the media at a rate that in some cases appears different to that of subsequent exponential growth. This apparent loss then recovery of viability on selective media has been observed for *E. coli* sublethally injured by treatment with a quaternary ammonium compound (Scheusner *et al.*, 1971), heat injured

L. monocytogenes, *S.* Typhimurium and *S. aureus* (Collins-Thompson *et al.*, 1973; Mackey and Derrick, 1982b), and for *S. aureus* injured by cold storage (Jackson, 1974). Mackey and Derrick (1982a) reported that for heat injured *S.* Typhimurium the choice of recovery medium had little effect on lag time estimates as resistance to a variety of selective media was regained by the end of the lag preceding cell multiplication.

4.1.1.4.2 Ground beef as a substitute for meat carcasses

The growth of bacteria causing spoilage or food poisoning is generally confined to the substrate surface (Wimpenny *et al.*, 1995). However, if the substrate is comminuted then surface bacteria may become distributed throughout the food matrix. Ground beef consists of trimmings from various cuts and is extensively handled and processed. It also has a large surface area which favours the growth of aerobic microorganisms (Jay, 1986). Consequently a comminuted product such as ground beef may contain high microbial loads. A preliminary survey conducted by the candidate on 4 retail mince packages yielded total viable counts in the range 5.90 to 7.30 log cfu.g^{-1}. Coliform counts were ≥ 110 cfu.g^{-1} and *E. coli* was present in all of the samples, ranging from 1.1 to 46 cfu.g^{-1}.

The natural microbiota contaminating ground beef complicates growth studies of mesophilic pathogens. The total viable count may reach maximum population density before the target organism has grown sufficiently for growth rate analyses. This overgrowth phenomenon has been referred to in the literature as the 'Jameson Effect' after Jameson (1962) reported that the presence of other organisms could affect the growth kinetics of *Salmonella*. The potential effects of natural microbiota has led researchers to sterilise, e.g. by roasting, beef prior to grinding (Abdul-Raouf *et al.*, 1993) or irradiating retail ground beef to reduce the microbial load (Palumbo *et al.*, 1997).

4.1.1.4.3 Antibiotic resistant strains

Inoculation studies are required to determine the growth, survival or death of pathogenic bacteria in foods. In the case of naturally contaminated foods this frequently requires the enumeration of a small

number of potentially sublethally injured cells in the presence of much larger numbers of other organisms (Blackburn and Davies, 1994a). Conventional methods of directly plating onto selective and/or differential media to enumerate pathogens may be inadequate as 'the degree of selectivity exerted is seldom sufficient to allow enumeration of any one type in the presence of closely related types' (Park, 1978). Injured cells do not develop well on selective media.

For successful studies based on inoculation of pathogens in foods it is necessary to overcome these deficiencies and use or even develop suitable media. The medium needs to suppress the majority of background flora and allow growth of injured cells while enabling easy detection and enumeration of target organisms. One way to overcome these difficulties is to use antibiotic-resistant mutants and a differential medium containing antibiotics in conjunction with normal total viable count media. The complication of naturally contaminating pathogenic flora in foods is then reduced or avoided. Antibiotic-resistant strains have been used successfully for enumerating pathogenic bacteria in foods (Mackey and Kerridge, 1988; Hart *et al.*, 1991; Blackburn and Davies, 1994a; Palumbo *et al.*, 1997; Wang *et al.*, 1997).

In using antibiotic resistant mutants to study survival and growth it is assumed that the mutants and the wild type behave similarly (Park, 1978). Growth rate studies on parent and mutant strains have, however, yielded conflicting results. Blackburn and Davies (1994a) demonstrated that some antibiotic-resistant strains have a slightly slower growth rate than their parent strain at near optimum temperatures. However, Park (1978), Hart *et al.* (1991) and Palumbo *et al.* (1997) found no significant difference in growth rates between parent and mutant strains.

4.1.1.4.4 Media

Non selective media containing antibiotics may not be suitable for examining low numbers of pathogenic bacteria in naturally contaminated foods as they may not inhibit the growth of non-target organisms. A differential or selective medium containing antibiotics may be required to distinguish target organisms from the resistant background flora. For

example, Eosin Methylene Blue (EMB) Agar containing antibiotics has been successfully used in recovering antibiotic-resistant strains of *E. coli* from stored foods (Blackburn and Davies, 1994a).

Numerous media are commercially available for the detection of *E. coli* in foods. Some are selective, for example Tryptone Bile Agar, while others are differential and selective, for example Violet Red Bile Agar, MacConkey Agar (MAC), and EMB. Selective media vary in their chemical composition and in their degree of selectivity. Media in the form of rehydratable preparations can be used to make solid agar plates and broths for purposes of enrichment or enumeration. Rehydratable dry-film plates such as the 3M™ Petrifilm™ range can be used as agar plate substitutes for the enumeration of bacteria.

Dry-film plates offer some advantages over traditional plate count and MPN methods, including: a built in grid for counting colonies; no media preparation and reduced plate storage requirements.

3M™ Petrifilm™ *E. coli* Count plates

3M™ Petrifilm™ *E. coli* Count plates (PECC) are rehydratable dry-film plates consisting of a 20cm^2 circular growth medium laminated between a hinged plastic and foam base. The medium consists of growth nutrients, violet red bile salts as the selective ingredient, the indicator dyes glucoronidase (5-bromo-4-chloro-3-indolyl-ß-D-glucoronide sometimes referred to as X-GLUC or BCIG) for *E. coli* and triphenyltetrazolium chloride for coliforms, housed in a guar gum base (manufacturers literature). Bacteria grow in the film and produce coloured colonies associated with gas bubbles formed from the fermentation of the lactose in the medium. Coliforms produce red colonies and glucuronidase positive *E. coli* produce blue colonies. Glucuronidase negative *E. coli* such as the EHEC group (Desmarchelier and Grau, 1997), will produce red colonies and additional or alternative identification measures are required.

Compared to traditional plate count and MPN methods the PECC offers all the advantages listed above as well as: results available in 24 (coliforms) to 48 (*E. coli*) hours; simultaneous detection of coliforms and

E. coli is possible and there is no requirement for confirmation of presumptive colonies on this medium (except for glucoronidase negative *E. coli* 0157:H7). PECC results are comparable to MPN and plate count methods for the enumeration of *E. coli* (Ingham and Moody, 1990; Matner *et al.*, 1990; Curiale *et al.*, 1991; Fitzgerald *et al.*, 1993) and for the enumeration of coliforms (Nelson *et al.*, 1984; McCallister *et al.*, 1988; Fitzgerald, 1993).

4.1.2 Assessment criteria for model evaluation

To date, no standard method or set of criteria has been published by which a model can be said to be validated (Ross, 1996). Traditionally predictive models have been assessed statistically by the 'goodness of fit' of the data used to generate them and pictorial comparisons of observed and predicted data. Residual plots are also used to identify any non-linearity or non-constant variance in a model. Ross (1996), however, proposed the use of the performance indices of bias and accuracy factors to extend evaluation of kinetic models by allowing comparison to other data. The bias and accuracy factors test, in effect, the hypothesis that the model under evaluation predicts the true mean, or that it represents it better than some other model (Ross, 1996). The bias and accuracy factors provide information similar to that of the observed versus predicted pictorial comparisons. The advantage of using these performance indices is that they are objective and quantitative measures that provide a simple means of reporting a readily interpretable assessment of model performance.

4.1.2.1 Observed versus predicted pictorial comparison

Regions of difference between model predictions and observed growth are easily *visualised* by plotting the observed values against predicted. Values are plotted on a log scale in order to normalise the variance encountered when measuring growth responses at the upper and lower limits (Ratkowsky *et al.*, 1991).

Perfect agreement between observed and predicted generation times results in a line of equivalence through the origin with a slope of one

(x=y). Points falling below this line predict generation times shorter, i.e. predicted growth is faster, than that observed. These predictions are considered 'fail-safe'. Conversely, points falling above the line, i.e. slower growth is predicted than that observed, are 'fail-dangerous'.

4.1.2.2 Residuals

Residuals are the *calculated* differences between the observed values of a response variable and the values predicted by a fitted equation. For models describing bacterial growth, observed and predicted responses can be compared by plotting the residuals, where growth rate is the response variable, against the predicted response, i.e. growth rate. The square-root of growth rate is often used to normalise variance.

On a residuals plot for growth rate, negative values below zero indicate the predicted response is faster than the observed response. Conversely, values above zero indicate the predicted response is slower than the observed response.

4.1.2.3 Bias and accuracy factors

With regard to generation time, the bias factor (Bf) is a measure of the relative average deviation of predicted and observed values and is expressed as the antilogarithm of the average of the logarithm of the ratio between the predicted and observed generation times:

$$Bf = 10^{(\Sigma \log(GTpredicted/GTobserved)/n)} \qquad \text{Eqn. (20)}$$

where $GT_{predicted}$ = predicted generation time, $GT_{observed}$ = observed generation time and n is the number of observations used in the calculation.

Careful interpretation of the bias factor is required. A bias factor of 1 indicates perfect agreement between observed and predicted generation times, however, under- and over-prediction will tend to 'cancel out' in this measure because the logarithm of the ratios will have opposite signs.

Therefore a bias factor of 1 is interpreted as indicating no *systematic* over- or under-prediction. Observations <1 indicate the model is usually predicting generation times shorter than observed and observations >1 as usually predicting generation times longer than observed. An under-prediction, i.e. $Bf<1$, may be regarded as "fail-safe' and an over-prediction, i.e. $Bf>1$, as 'fail-dangerous'. Judgement must be exercised when assessing a model by this index as a low bias factor (i.e. <1) whilst indicating the model as 'fail-safe' also suggests a level of conservatism that may render the model not practically useful.

The accuracy factor (Af):

$$Af = 10^{(\Sigma\,|\log(GTpredicted/GTobserved)|/n)} \qquad \text{Eqn. (21)}$$

where the terms are as previously defined, provides an indication of the spread of the results about the prediction. An accuracy factor of 1 implies perfect agreement between observed and predicted values. Values larger than 1 indicate a difference between observed and predicted generation times. The larger the value, the less accurate is the average estimate.

4.1.3 Objectives

In this chapter the square-root type model for *E. coli* developed in Chapter 3, Eqn. (18), is evaluated in comparison to laboratory generated data and data collated from the literature. A collaborative study for an industry evaluation of the square-root type model for *K. oxytoca* developed in Chapter 3, Eqn (19), is also discussed.

4.2 MATERIALS AND METHODS

4.2.1 Model evaluation: laboratory studies

Two techniques for recovery of *E. coli* inoculated into ground beef in the presence of naturally occurring microbiota were considered: (i) a commercial selective medium and (ii) using an antibiotic resistant strain with media containing antibiotics. A preliminary investigation of

differences between the growth characteristics of the parent and antibiotic resistant strain was performed.

Inoculation studies which monitored the growth of *E. coli* in ground beef, either axenic or with low numbers of naturally occurring microbiota, at variable temperature were undertaken to enable a laboratory evaluation of Eqn. (18). Observed generation time estimates were compared to model predictions using bias and accuracy factors (S. 4.1.2.3).

4.2.1.1 Method development

4.2.1.1.1 A commercial dry-film technique for enumeration of *E. coli*

OVERVIEW

The recovery of *E. coli* M23 on PECC at near limiting growth conditions in a broth system was investigated and compared to recovery on PCA, and EMB. A stationary phase inoculum was inoculated into BHI broth of a_w 0.961 and placed at 12 (±0.1)°C. Numbers were monitored by viable count. After 267 hours, and a 3 log reduction in numbers, the inoculated broth culture was adjusted to provide more favourable conditions for growth: 20°C and a_w 0.979. Linear regression analysis was used to evaluate differences in the recoverability of the three media types and to determine the suitability of PECC for laboratory performance evaluations.

METHOD

A stationary phase inoculum, (S. 2.1.2.1, primary inoculum) of *E. coli* M23 was prepared in BHI, with a measured a_w of 0.997. The stationary phase inoculum was serially diluted in PW to provide $10^{5\text{-}6}$ cfu.mL^{-1}.

25μL of the diluted inoculum was dispensed into 50mL of BHI containing 6.5% NaCl (a_w 0.961) and equilibrated to 12.0 (±0.1°C). The inoculated broth was placed in a shaking waterbath and maintained at 12°C and a_w 0.961 for 267 hours. The broth culture was then shifted to a 20°C waterbath and the a_w of the culture raised to a_w 0.978 by addition of sterile BHI broth in a ratio of 1 to 1.

Prior to inoculation into the test broth, the total viable aerobic count of the inoculum was determined by spread plating of 0.1mL aliquots on PCA and incubating plates at 37°C for 24 hours.

Immediately post inoculation and at regular time intervals, aliquots of the test broth were removed and, where required, serially diluted in PW. Viable counts were determined on PCA and EMB agar plates or PECC. For determinations on EMB and PCA a spread plate method was used (S. 2.1.3.1.2) and plates were incubated at 35°C.

For determinations on PECC, 1mL aliquots of appropriate dilutions were dispensed onto the centre of the bottom film of duplicate PECC plates. The plastic cover film was gently lowered onto the sample to prevent entrapment of air bubbles. The sample was distributed evenly by gentle downward pressure of a plastic spreader supplied by the manufacturer. Plates were left on the bench top for 5 minutes to permit solidification of the gel. Plates were incubated in a horizontal position at 35°C and colonies counted after 48±2 hours.

Log viable count from each of the three methods were plotted against time.

4.2.1.1.2 Development of streptomycin resistant *E. coli* M23

A spontaneous streptomycin resistant mutant of *E. coli* M23 was isolated using a modification of the method of Park (1978) with a ten-fold reduction in the concentration of antibiotic used. A stationary phase inoculum of parent *E. coli* M23 was prepared (S. 2.1.2.1, primary inoculum) in 100mL of NB in a 250mL conical flask. 100mL of Nutrient Broth containing 200μg.mL^{-1} Streptomycin, NB-2S, was added aseptically to the 14 hour culture. At 24, 48 and 96 hours a loopful of the broth was streaked onto Eosin Methylene Blue Agar containing 100μg.mL^{-1} streptomycin, EMB-S, then incubated at 37°C for 48 hours. A representative colony was picked from the EMB-S and streaked onto PCA and incubated at 37°C for 24 hours to obtain a pure culture. The notation "SR-M23" was designated for the isolate.

E. coli SR-M23 was stored cryogenically (A. 9.1.2) and resuscitated when required (A. 9.1.3). *E. coli* SR-M23 was routinely tested for antibiotic resistance by streaking onto EMB-S and tested for antibiotic dependency by streaking onto PCA.

4.2.1.1.3 Growth rate comparison

OVERVIEW

The growth rate of parent *E. coli* M23 and mutant streptomycin resistant *E. coli* M23 were compared at four temperatures. Stationary phase cells were inoculated into fresh BHI and incubated with shaking. Growth was monitored turbidimetrically and growth curves analysed using non-linear regression.

METHOD

Stationary phase inocula of *E. coli* M23 and *E. coli* SR-M23 in BHI (at 35 (±0.1)°C) were prepared as described in S. 2.1.2.1 (primary inoculum).

0.5mL of each inoculum was dispensed into a series of BHI broths (50mL) which were pre-warmed to the appropriate temperature, and placed in shaking waterbaths at 20(±0.1), 25(±0.1), 30(±0.1) and 35(±0.1)°C.

Growth was monitored turbidimetrically (S. 2.1.3.1.1) and curves analysed using non-linear regression (S. 2.1.3.2) to estimate values for generation time.

4.2.1.2 *E. coli* in ground beef

OVERVIEW

The validity of Eqn. (18) was tested by laboratory inoculation studies of *E. coli* SR-M23 in freshly ground, axenic beef tissue at 10 (±0.1), 12 (±0.1), 15 (±0.1) and 20 (±0.1) °C. The effect of naturally contaminating mince flora on the growth of SR-M23 was also examined by inoculations into a mixture of retail mince and axenic ground beef (1:1000). This achieved sufficient dilution of the naturally occurring microbiota present in the retail ground beef, thus enabling growth of SR-M23 to be monitored.

Growth of SR-M23 in the axenic and retail mix and the total aerobic microbiota in the retail mix was monitored in the inoculated samples by viable count. Agreement between the observed generation time estimates, from linear regression analysis of the growth curves, and predictions from a number of *E. coli* growth models was examined using bias and accuracy factors (S. 4.1.2.3).

METHOD

A stationary phase inoculum (S. 2.1.2.1 primary inoculum) of *E. coli* SR-M23 was prepared in BHI at 20 (±0.1)°C. The inoculum was diluted 100 fold in PW to provide a "washed" cell suspension of ~10^{5-6} cfu.mL^{-1}, i.e. there was minimal transfer of nutrients.

Preparation of ground beef

Axenic ground beef

The surface layer, ~10mm, of a large topside beef roast (1.5 to 2.0 kg), purchased from a local supermarket, was aseptically removed using sterile knives. The 'sterile' inner tissue was cut into portions then ground using an autoclaved metal hand mincer. The axenic ground beef was stored at 2°C for up to 10 hours prior to use. Total viable count was determined prior to inoculation with the test organism to ensure the material was free from contamination.

Naturally contaminated (low-level) ground beef

Ground beef with a low level of naturally contaminating microbiota (i.e. 10^{2-3} cfu.g^{-1}) was prepared by adding retail supermarket mince (10% fat) to prepared axenic ground beef in the ratio 1:1000.

1mL of the diluted inoculum was pipetted onto the surface of 100g of prepared ground beef and mixed through by hand (sterile gloves) in a sterile stainless steel mixing bowl to give a final concentration of *E. coli* SR-M23 of ~10^{3-4} cfu.g^{-1}. The hand mixed ground beef was then passed through a sterile hand mincer to ensure uniform distribution of bacteria and to provide a surface area similar to that of retail product.

5g portions of the seeded mince were placed into individual wells (12) of a sterile multi-welled culture plate. Well plates were sealed with O_2 permeable film, placed inside 2 plastic bags which were sealed and immersed in waterbaths at the appropriate temperature. At regular intervals duplicate 5g portions of inoculated ground beef were removed and placed in separate stomacher bags. 45mL of PW was added and the samples stomachered for 2 minutes. The homogenate was serially diluted in PW and 0.1mL aliquots of appropriate dilutions were surface plated onto PCA and EMB-S agar plates. PCA plates were incubated at 22°C and colonies counted after 48 hours to provide an estimate of total viable count. EMB-S plates were incubated at 35°C and colonies counted after 24 hours to estimate the viable count for *E. coli* SR-M23.

Generation times were estimated using linear regression (S. 2.1.3.3). Bias and accuracy factors (S. 4.1.2.3) were calculated to compare the observed generation times in the ground beef samples to predictions of growth of *E. coli* generated by Eqn. (18), Food MicroModel (FMM), Pathogen Modeling Program Version 5.0 (PMP) and a square-root type model described in Ross (1993).

FMM and PMP are software packages containing a range of predictive models which can estimate the effects of multiple variables on the growth and survival of foodborne pathogens. PMP is provided free of charge by the Microbiological Food Safety Research Unit of the United States Department of Agriculture (USDA). FMM is available for an annual fee from the Leatherhead Food Research Association, Surrey, England.

4.2.2 Model evaluation: literature

A literature based performance evaluation of Eqn. (18) was undertaken using bias and accuracy factors as indicators of model performance. The performance of Eqn. (18) was compared with other models in the literature for *E. coli* growth. Growth rate data were collated from the published scientific literature and assessed for inclusion in the performance evaluations. Where necessary, correction factors were applied to allow comparison between data generated with different methodologies (*see* S. 3.1.3).

4.2.2.1 Collation of growth rate data

A comprehensive search of the literature for growth rate data for *E. coli* was performed and the data collated into spreadsheets. A total of 37 sources (33 publications, 2 personal communications and 2 data sets generated by the candidate) were identified. The number of observations from each source ranged from 1 to 281.

In the absence of tabulated results it was often necessary to estimate values from copies of graphs and to calculate growth data either by linear or non-linear regression. If the manuscript did not contain values for lactic acid concentration, pH or a_w, they were obtained from other literature sources or estimated. Lactic acid concentrations were estimated for all the meat data using the values of Grau (1981) for high and low pH beef as a guide. The pH values of laboratory media were obtained from media specifications detailed in Atlas (1993). The a_w values for some laboratory culture media were obtained from Chirife *et al.* (1982). Where necessary %NaCl values presented in Atlas (1993) were converted to a_w using the tables of Chirife and Resnik (1984). A summary, including details of the source, growth conditions and strains used, for each of the literature data sets is presented in A. 9.4.4 and A. 9.4.5.

To prevent undermining of model performance by comparison against poor quality data, generation time data from the literature were edited. Data were excluded if:

- The growth media used could retard growth, e.g. minimal media or media containing antibiotics.
- In the case of complex laboratory media, anaerobic conditions for growth prevailed (anaerobic conditions in foods were included).
- In the absence of tabulated data there were insufficient data points for linear or non-linear regression analysis for generation time estimation.
- In the case of foods, there were parameters not covered by the predictive models which may exert an effect, e.g. modified atmosphere packaging.
- Growth data was generated in conditions outside of those used to create the model. For Eqn. (18) the conditions covered by the model

were for the ranges: 7.6 to 47.4°C, a_w 0.958 to 0.999 and pH 4.13 to 8.28.

4.2.2.2 Assessment of Eqn. (18)

Predictions from Eqn. (18) were compared to the collated literature data using bias and accuracy factors as indicators of model performance. Predictions from PMP were also compared to the literature data. PMP model limits were 8.9 to 42°C, a_w 0.970 to 0.997 and pH 4.5 to 8.5. Similarly, the literature data were compared to a second order polynomial equation fitted to *E. coli* growth data compiled from FMM. The polynomial equation was designed as a proxy for the FMM software package. Inserting a formula into the established Excel worksheet containing all the literature data was deemed a more efficient process than performing manual entries for each of the growth data. Details on development of the proxy equation, herein referred to as *p*FMM-*Ec*, can be found in Ross, 1999; pp 75-76. The reliability of the proxy model was assessed against the genuine FMM data using bias and accuracy factors, which were 1.0008 and 1.004 respectively. The deviation in the bias and accuracy factors from 1, indicative of a perfect fit, derives mainly from rounding errors. Thus, *p*FMM-*Ec* is *essentially* a perfect copy of the genuine FMM *Escherichia coli* model. None-the-less, the designation *p*FMM-*Ec* will continue to be used in this thesis as genuine Food MicroModel predictions have been used elsewhere (these are referred to as FMM). For *p*FMM-*Ec* the model limits were: 10 to 30 °C, a_w 0.960 to 1.000 and pH 4.5 to 7.5.

The observed growth rate responses from the literature were also compared to those predicted by Eqn. (18) using observed versus predicted pictorial comparisons and plotting residuals.

4.2.2.2.1 Comparison to data presented in Sutherland *et al.* (1995)

Sutherland *et al.* (1995) undertook a comparison of predicted growth responses for *E. coli* from 2 polynomial based models; one based on a modified Gompertz model presented in that paper and the other based on the D-model of Baranyi *et al.* (1993a), with those reported in the literature

for growth in laboratory media and food. The literature data presented in that paper is a sub-set of the data collated in this thesis. Predictions from Eqn. (18) and PMP were generated for some of the data of Sutherland *et al.* (1995). Predictions from all of the models were compared to the data using bias and accuracy factors.

It was not possible to obtain all the references used by Sutherland *et al.* (1995), thus comparisons were not performed on all data presented in that paper. For Eqn. (18), it was necessary to estimate a_w and [LAC] values, and for PMP it was necessary to estimate a_w values. Data for growth of *E. coli* in solid dairy foods was not considered due to the lack of specificity of some pH and temperature values which were reported as ranges. The data used for comparisons is listed in A. 9.4.6.

4.2.3 Model evaluation: industry

The materials and methods used in the carcass chilling study are detailed in Ross (1999). To summarise from that document, exponential phase cultures of *K. oxytoca* were deliberately inoculated onto carcasses and the change in numbers followed during normal weekend chilling operations. a_w measurements at the carcass surface were taken using the method of Salter (1998), at times which coincided with sampling for viable count determinations. Temperature data were collected with data loggers at 15 minute intervals. Viable counts of *K. oxytoca* were inferred from coliform counts determined on PECC. Using 'environmental history integration' the observed growth under those conditions was compared to predictions from Eqn. (19).

4.3 RESULTS

4.3.1 Model evaluation: laboratory

4.3.1.1 Method development

4.3.1.1.1 3M™ Petrifilm™ *E. coli* Count plates for recovery of *E. coli*

PECC was found to be more selective against *E. coli* cells than PCA and EMB under conditions which lead to cell death, and also to inhibit recovery

in the early stages of growth at more favourable conditions (Fig. 4.1). Raw experimental data are presented in A. 9.4.7.

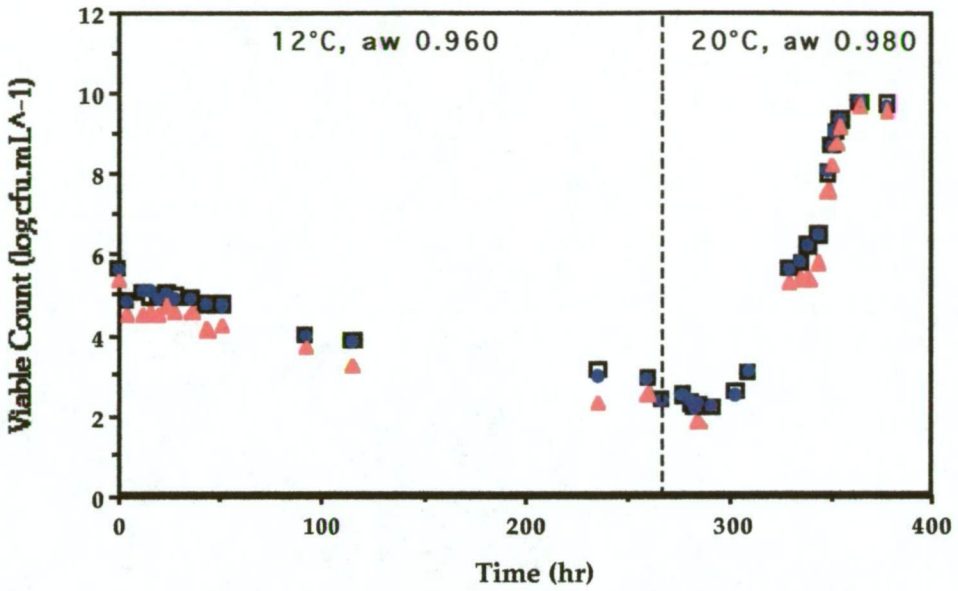

Figure 4.1: Assessment of efficacy of media for the recovery of *E. coli* M23 at growth limiting conditions followed by a shift, marked (---), to conditions allowing growth, where: □ = Plate Count Agar, ● = Eosin Methylene Blue Agar and ▲ = 3M™ Petrifilm™ *E. coli* Count plates.

Under conditions which lead to cell death, 12°C and a_w 0.960, PECC counts were consistently lower, by approximately 1 log cfu.mL^{-1}, than the PCA and EMB counts.

After 267 hours, and a 3 log reduction in numbers, the inoculated broth culture was adjusted to enable *E. coli* growth; i.e. 20°C and a_w 0.979. Recovery of *E. coli* M23 on PECC plates remained consistently lower than on PCA or EMB in the lag and exponential phases but more closely approached the level of recovery on those media when the population reached stationary phase.

Linear regression of the data in the 0 to 267 hour range, where conditions were 12°C and a_w 0.960, was undertaken using SAS PROC.GLM (SAS Institute Inc., Cary, N.C.) (Table 4.1).

Table 4.1: Linear regression equations for recovery of *E. coli* M23 at 12°C, a_w 0.960 on different media.

Media	Linear regression equation
□ PCA	y = -0.515777 + 0.009473x
● EMB	y = -0.519420 + 0.009473x
▲ PECC	y = -0.470680 + 0.009473x

The slope of the regression lines were found not to be identical, however they were not significantly different (P=0.7908). The slope of each line was fixed to the common slope value to determine if the *x* axis intercepts were different. This analysis revealed that the intercepts were significantly different (P=0.0001). The data for PCA and EMB share a similar intercept that is different from the PECC data.

4.3.1.1.2 Growth rate comparison between *E. coli* M23 and streptomycin resistant *E. coli* M23

The parent and mutant strain of *E. coli* M23 exhibited a similar, but not identical, growth rate response to temperature (Fig. 4.2). Linear regression analysis, using Cricket Graph v.1.3.2, was used to provide a line of best fit through the four datum points for each strain. T_{min} values were determined from the fitted equations (*see* Table 4.2). Raw experimental data are presented in A. 9.4.8.

Table 4.2: Linear *regression equations* and T_{min} for growth curves of *E. coli* M23 and *E. coli* SR-M23 at various temperatures.

Data Set	Linear regression equation	T_{min}
■ *E. coli* M23	y = - 6.7958e-3 + 6.0576e-3x (R^2 = 0.941)	1.5
● *E. coli* SR-M23	y = - 2.9652e-2 + 6.6604e-3x (R^2 = 0.951)	4.4

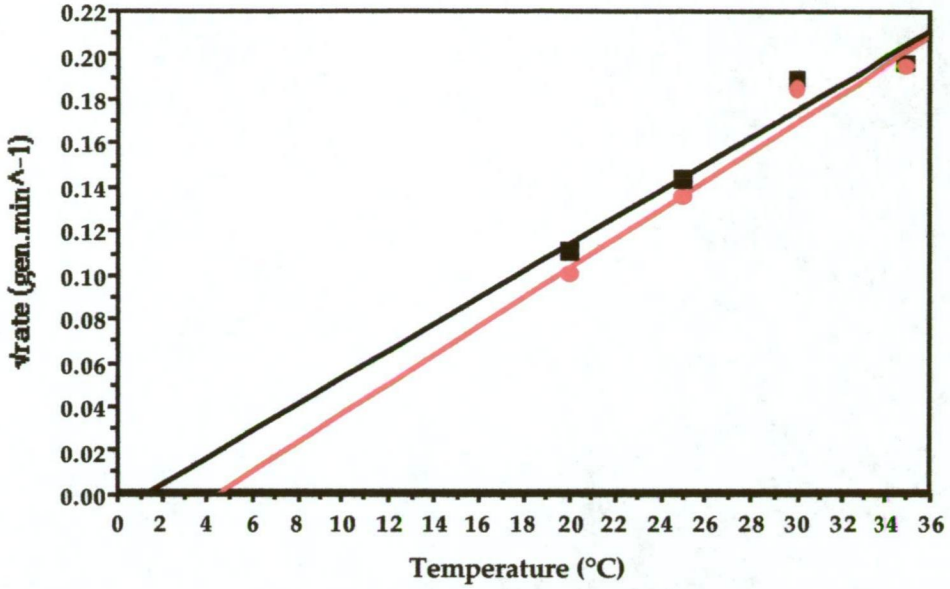

Figure 4.2: Growth characteristics of *E. coli* M23 (■) and streptomycin resistant *E. coli* M23 (●) in Brain Heart Infusion Broth at various temperatures.

4.3.1.2 Laboratory performance evaluation: *E. coli* in ground beef

E. coli SR-M23 was inoculated into freshly ground, axenic beef tissue and into a mixture of retail mince and axenic ground beef (1:1000). Growth was monitored by viable count and generation times calculated (Table 4.3).

Table 4.3: Observed and predicted generation times from various models for growth of *E. coli* SR-M23 in ground beef with and without natural biota at various temperatures where pH=6.0, aw=0.997 and [LAC] is estimated at 100mM.

	Generation Time (hours)					
	Observed		Predicted			
°C	Axenic	Retail mix	Eqn. (18)	Ross (1993)	PMP	FMM
10	6.6 EST	NSG	9.8	8.9	5.9	9.5
12	3.9	6.4	5.4	4.9	4.1	6.0
15	2.5	3.0	2.8	2.5	2.5	3.2
20	1.5	1.4	1.3	1.2	1.2	1.3

NSG=Non-sustainable growth, EST=Estimate

The total aerobic viable count was also monitored in the retail mix. Generation times for *E. coli* SR-M23 were compared to predictions from a model generated by the candidate, a model from the literature, (Ross, 1993) which had a lower limit of pH 6.4, and two commercial modelling packages; PMP and FMM.

Using bias and accuracy factors (S. 4.1.2.3), the predictions of the models for growth of *E. coli* SR-M23 in axenic ground beef and the retail / axenic mixture were assessed by comparison to observed growth rate (Table 4.4).

Table 4.4: Bias and accuracy factors for comparison of observed growth of *E. coli* SR-M23 and predicted growth of *E. coli* in axenic ground beef and a retail mix (best bias and accuracy marked in bold).

	Axenic (*n*=3)		Retail/Axenic Mix (n=3)		All (n=6)	
Model	**Bf**	**Af**	**Bf**	**Af**	**Bf**	**Af**
Eqn. (18)	1.20	1.21	0.87	1.15	**1.04**	1.22
Ross 1993	1.08	1.16	0.82	1.22	0.91	**1.19**
PMP	**0.93**	**1.10**	0.77	1.30	0.85	**1.19**
FMM	1.25	1.31	**0.98**	**1.07**	1.08	**1.19**

As there were insufficient data points for the 10°C growth curve, the generation time calculated by linear regression is an estimate (Table 4.3); only 3 points were used in the calculation due to curvature in the exponential portion of the growth curve. This determination was not used in bias and accuracy calculations listed in Table 4.4.

E. coli SR-M23 reached MPD, around 10^7 cfu.g-1, at similar times in the 20 and 15°C trials; at approximately 17 and 30 hours respectively (Fig. 4.3 and Fig. 4.4). The total aerobic viable count also appeared to reach MPD at these times.

At 12°C (Fig. 4.5), the MPD reached by *E. coli* SR-M23 in the retail mix was approximately 1 to 1.5 logs lower than its axenic counterpart. MPD was reached at approximately 40 hours under all three conditions.

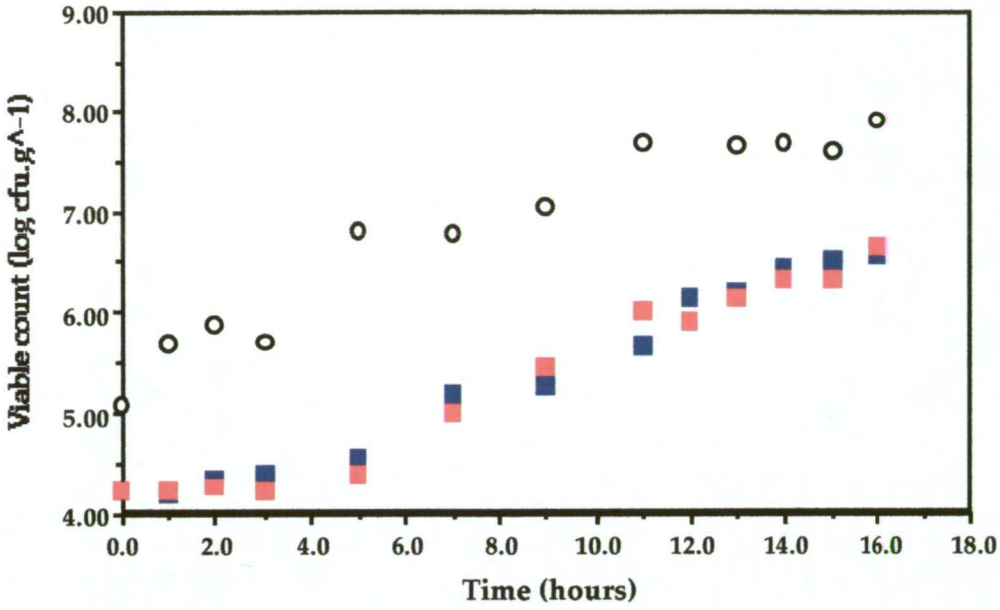

Figure 4.3: Growth of *E. coli* SR-M23 (■) at 20°C in axenic ground beef and of *E. coli* SR-M23 (■) and total aerobic viable count (○) in retail mix at 20°C.

At 10°C, *E. coli* SR-M23 was unable to sustain growth in the retail mix (Fig. 4.6). In comparison, *E. coli* SR-M23 was able to sustain growth in the axenic ground beef at the same temperature.

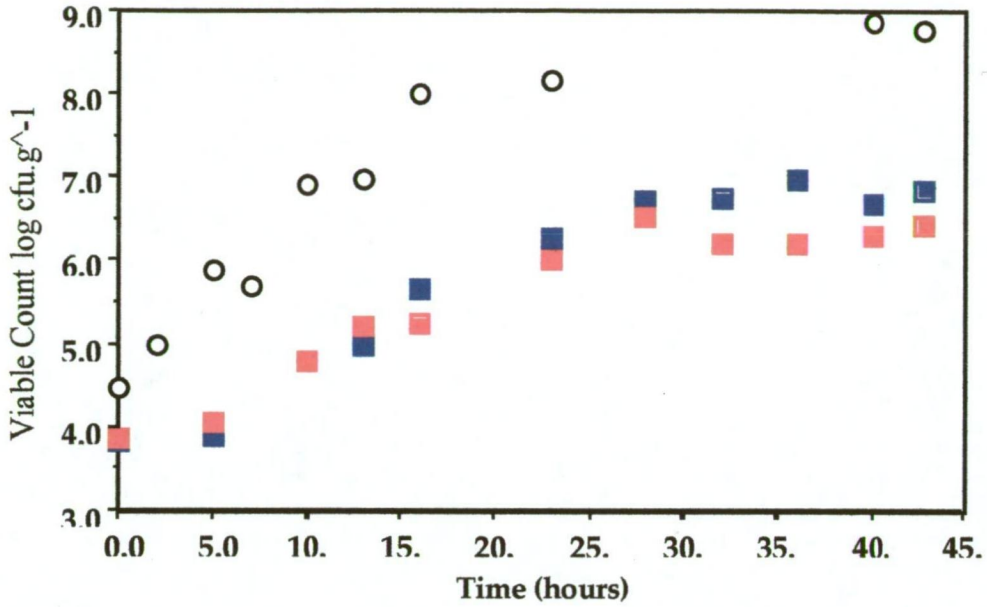

Figure 4.4: Growth of *E. coli* SR-M23 (■) at 15°C in axenic ground beef and of *E. coli* SR-M23 (■) and total aerobic viable count (○) in retail mix at 15°C.

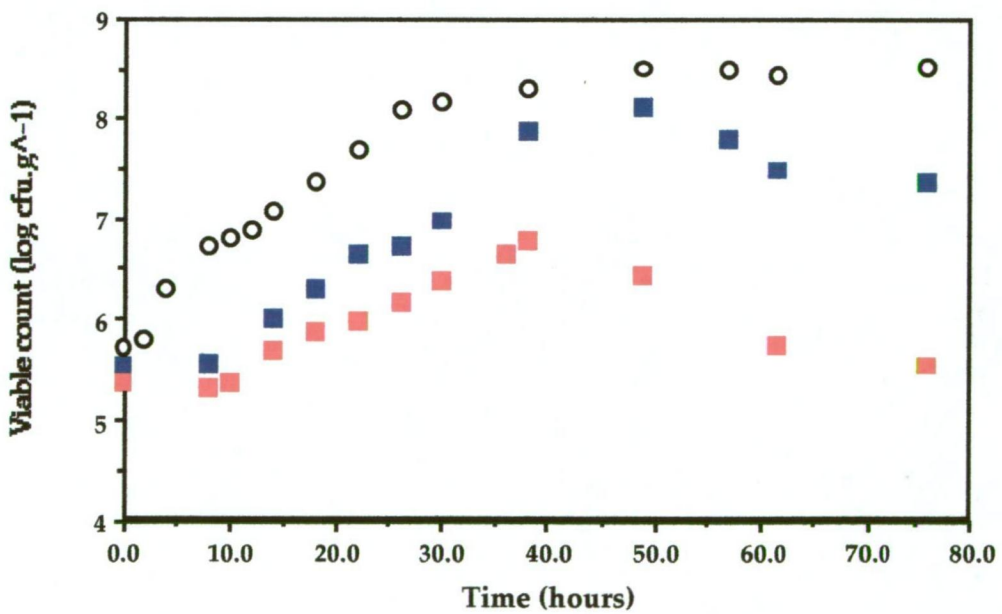

Figure 4.5: Growth of *E. coli* SR-M23 (■) at 12°C in axenic ground beef and of *E. coli* SR-M23 (■) and total aerobic viable count (○) in retail mix at 12°C.

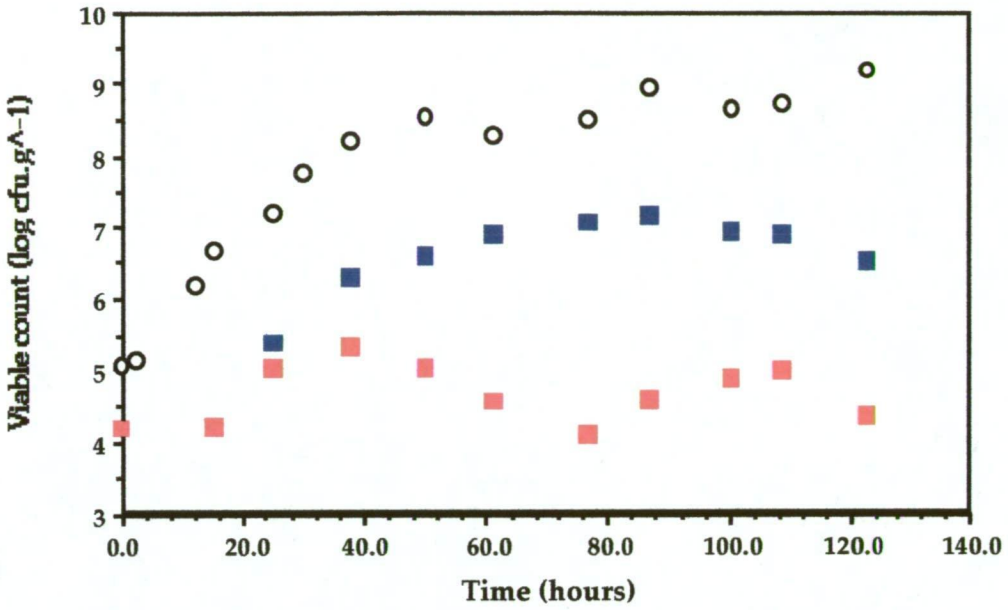

Figure 4.6: Growth of *E. coli* SR-M23 (■) at 10°C in axenic ground beef and of *E. coli* SR-M23 (■) and total aerobic viable count (○) in retail mix at 10°C.

4.3.2 Model evaluation: literature

1217 generation time values for growth of pathogenic and non-pathogenic *E. coli* in liquid media and foods were edited according to the criteria outlined in S. 4.2.2.1. The edited data comprised 1003 growth rate estimations. The edited data were separated into categories of complex laboratory media (n=825) and food (n=178). The food category was further separated to meat only (n=130) and 'other foods' (n=48). For the food category, anaerobic data were included in analyses as assessment of the atmospheric condition within comminuted products etc. is difficult. The surface is aerobic but it is likely that the interior may be anaerobic.

The observed growth rate data from the literature were compared to predictions from Eqn. (18) using graphical comparisons of $\log(GT_{observed})$ versus $\log(GT_{predicted})$ (S. 4.1.2.1), by plotting residuals ($\sqrt{r}_{observed}$-$\sqrt{r}_{predicted}$) against the $\sqrt{r}_{predicted}$ (S. 4.1.2.2) and calculation of bias and accuracy factors (S. 4.1.2.3). A second model for *E. coli*, a polynomial equation fitted to *E. coli* growth rate predictions from Food MicroModel (*p*FMM-*Ec*) was also

compared to the literature data using bias and accuracy factors. A sub-set of the literature data that was presented in Sutherland *et al.* (1995) was also used for comparisons to Eqn. (18) and *p*FMM-*Ec* using bias and accuracy factors.

4.3.2.1 Observed versus predicted pictorial comparisons

The predicted values from Eqn. (18) in comparison to the literature values are presented in Figure 4.7 for 'media', and Figure 4.8 for 'meat' and 'other foods'.

Much of the literature data for 'media' falls near the line of equivalence, i.e. where observed and predicted values are in perfect agreement. There appear to be fewer points in the 'fail-dangerous' area, i.e. above the line of equivalence. At the longer generation times the model predictions, whilst 'fail-safe', appear quite conservative.

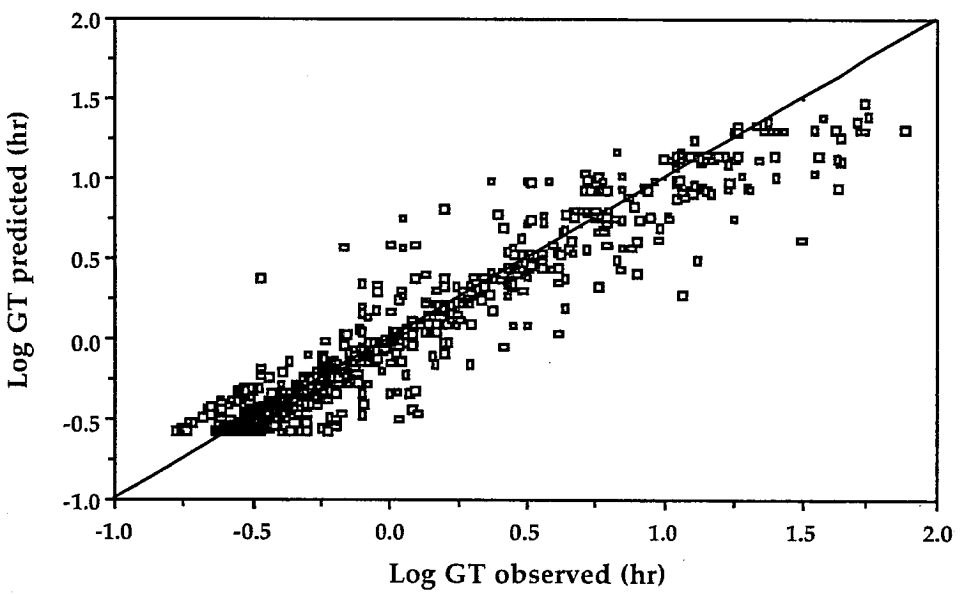

Figure 4.7: $Log(GT_{observed})$ versus $log(GT_{predicted})$ for the growth responses of *E. coli* reported in the published literature for 'media' and predictions from Eqn. (18).

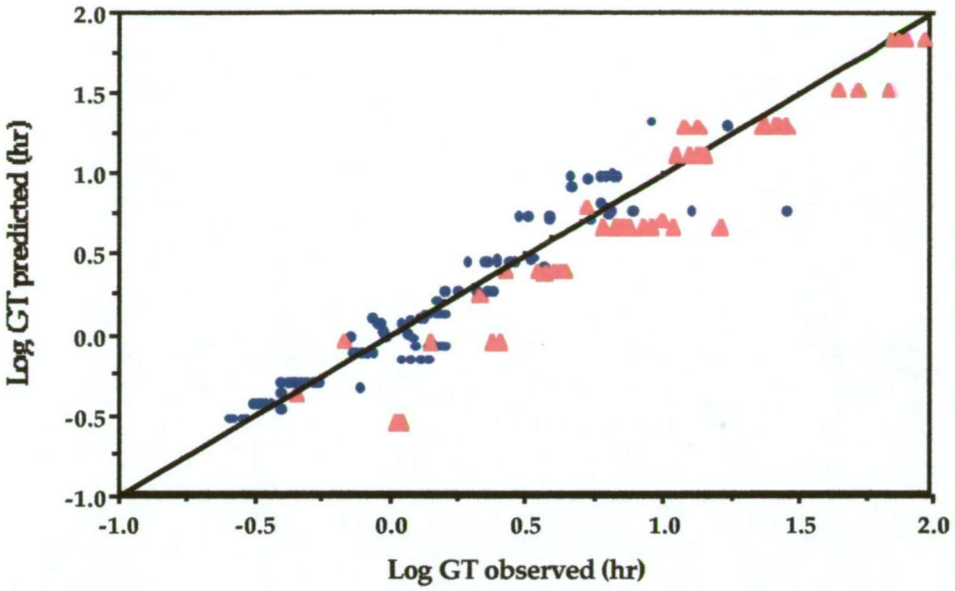

Figure 4.8: $Log(GT_{observed})$ versus $log(GT_{predicted})$ for the growth responses of *E. coli* reported in the published literature and predictions from Eqn. (18) where: ●='meat' and ▲='other foods.

For 'meat, much of the literature data falls alongside the line of equivalence, and most of the points appear closer than that observed for media. In comparison, the points for 'other foods' generally fall below the line of equivalence.

4.3.2.2 Residuals plots

The residuals plot for all the literature data compared to predictions from Eqn. (18) shows little systematic deviations between observations and predictions, although the degree of scatter is slightly wider at longer generation times (Fig. 4.9).

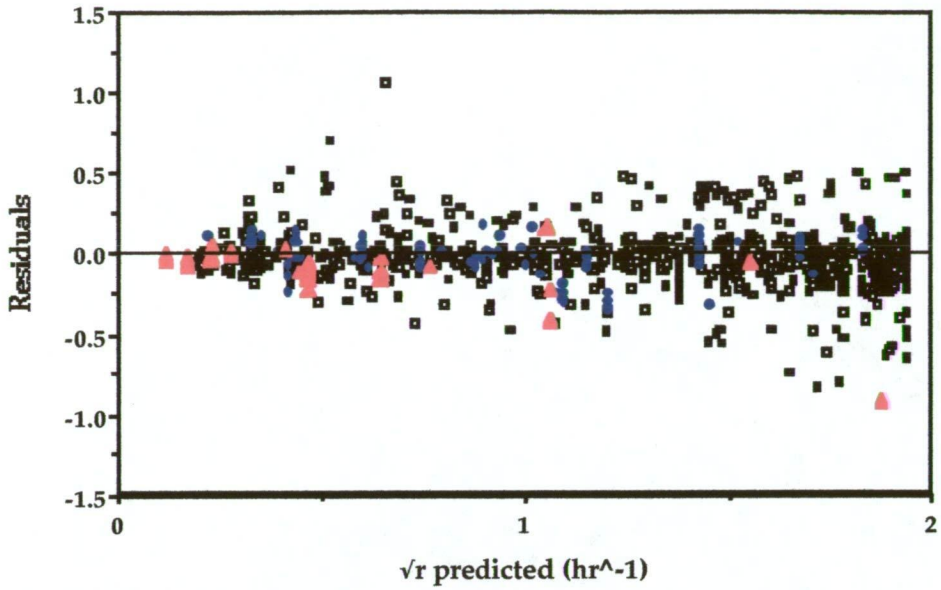

Figure 4.9: The residual plot ($\sqrt{r}_{observed}$-$\sqrt{r}_{predicted}$) against $\sqrt{r}_{predicted}$ for comparison of the growth responses of *E. coli* reported in the published literature for media and predictions from Eqn. (18) where: □='media', ●='meat' and ▲='other foods.

4.3.2.3 Bias and accuracy

Eqn. (18) performed better than PMP and *p*FMM-*Ec* for most of the data groups (Table 4.5).

Table 4.5: Bias and accuracy factors for growth of *E. coli* with data collated from the literature compared to Eqn. 18, PMP and a proxy model for Food MicroModel, *p*FMM-*Ec* (best bias and accuracy marked in bold).

	Eqn. (18)			PMP			*p*FMM-*Ec*		
Category	***n***	**B*f***	**A*f***	***n***	**B*f***	**A*f***	***n***	**B*f***	**A*f***
All-unedited	1217	0.85	1.42	879	0.81	**1.39**	617	**1.07**	1.54
All-edited	1003	**0.92**	**1.29**	802	0.87	1.30	509	1.16	1.46
Media	825	**0.93**	**1.28**	642	0.91	1.27	376	1.34	1.46
All Food	178	**0.86**	**1.36**	160	0.73	1.42	139	0.77	1.47
Meat	130	**0.97**	**1.26**	128	0.80	1.30	110	0.75	1.52
Other Foods	48	0.62	1.71	32	0.50	2.01	29	**0.84**	**1.27**

However, when the food category was divided into 'meat' and 'other foods', Eqn. (18) performed best for 'meat' and *p*FMM-*Ec* for

'other foods'. Foods other than meat consisted primarily of milk products. It is for this category only that *p*FMM-*Ec* out-performed Eqn. (18) for each of the performance indices. For the unedited data set *p*FMM-*Ec* outperformed Eqn. (18) for the bias factor only. PMP had performance indices close in number to Eqn. (18) for most data sets except for food.

To investigate whether the models perform better at a particular generation time, data were grouped into the categories of: $GT_{observed}$ <1 hour, $GT_{observed}$ 1-5 hour and $GT_{observed}$ >5 hour. Bias and accuracy factors were calculated and the results presented in (Table 4.6).

Table 4.6: Bias and accuracy factors for generation time subsets of edited data for growth of *E. coli* obtained from the literature compared to Eqn. 18 and a proxy for Food MicroModel (best bias and accuracy marked in bold)

Category	GT set (h)	Eqn. (18)			PMP			*p*FMM-*Ec*		
		n	*Bf*	*Af*	*n*	*Bf*	*Af*	*n*	*Bf*	*Af*
All-edited	<1	597	**0.97**	**1.19**	488	0.95	**1.19**	197	1.23	1.40
	1-5	247	**0.91**	**1.39**	210	0.84	1.40	221	1.15	1.59
	>5	159	0.76	1.56	104	0.61	1.70	91	**1.03**	**1.39**
Media	<1	546	**0.97**	**1.20**	439	0.96	**1.20**	166	1.36	1.41
	1-5	164	**0.98**	1.36	128	0.97	**1.33**	143	1.46	1.59
	>5	115	0.74	1.57	75	0.61	1.70	67	**1.08**	**1.41**
All Food	<1	51	**1.05**	**1.13**	49	0.93	1.15	31	0.72	1.38
	1-5	83	**0.78**	**1.45**	82	0.68	1.52	78	0.75	1.59
	>5	44	0.82	1.54	29	0.60	1.69	24	**0.91**	**1.47**
Meat	<1	49	**1.05**	**1.11**	49	0.93	1.15	31	0.72	1.38
	1-5	64	**0.88**	**1.31**	64	0.74	1.39	64	0.72	1.60
	>5	17	1.09	1.57	15	0.68	1.50	15	**0.98**	**1.46**
Other Food	<1	2	**1.11**	**1.01**	0			1	1.36	**1.01**
	1-5	18	0.49	2.03	17	0.46	2.18	14	**0.83**	**1.26**
	>5	28	0.71	1.52	15	0.55	1.84	11	**0.82**	**1.32**

Eqn. (18) performed best for generation times in the <1 and 1 to 5 hour category. The 'other foods' category was the exception, with poor bias and accuracy factors for the 1 to 5 hour category. For meat, Eqn. (18) had a similar bias factor for each group and the accuracy factor deteriorated with increasing generation time.

The best predictions for PMP were for the <1 hour category. For media PMP had similar bias and accuracy factors to Eqn. (18) for the <1 and 1 to 5 hour categories. Bias and accuracy factors deteriorated with increasing generation time for all categories.

For *p*FMM-*Ec*, the best bias factors were generally in the >5 hour group with the exception of the 'other foods' category for which the best bias factor was observed in the <1 hour group. Accuracy factors were all higher than 1.27. The worst accuracy factor for each category were mostly found in the 1 to 5 hour group.

Overall, Eqn. (18) appeared to predict better at generation times <5 hours. PMP predicted best for the <1 hour category. *p*FMM-*Ec* was consistently better at generation times >5 hours.

4.3.2.3.1 Comparison to data presented in Sutherland *et al.* (1995)

Bias and accuracy factors were used to compare the predictions from a number of models for *E. coli* growth rate to some of the literature data presented in Sutherland *et al.* (1995) (*see* Table 4.7). The models compared were: a modified Gompertz model (presented in Sutherland *et al.*(1995); a polynomial model based on the D-model of Baranyi *et al.* (1993a); PMP and Eqn. (18).

Generally, Eqn. (18) had the best bias and accuracy factor for each of the subsets of data for meat. For media, the performance of Eqn. (18) was less consistent. Overall, Eqn. (18) performed better than the other models for each category with the best bias factors for all three groups and the best accuracy factor for the 'meat' and 'media and meat' categories.

The overall performance of the other models was similar, although the modified Gompertz model of Sutherland *et al.* (1995) generally performed better with media. The bias factor for PMP was generally low, often <0.7, in all categories, suggesting that this model seriously under-predicts generation time and is too conservative, i.e. too 'fail-safe'.

Table 4.7: **Bias and accuracy factors: comparison of the modified Gompertz model of Sutherland *et al.*. (1995), the Baranyi D-model, PMP (the number of data compared is marked after slash) and Eqn. 18 to literature data for growth of *E. coli* (presented in Sutherland *et al.*, 1995). Best bias and accuracy marked in bold.**

		Eqn. (18)		PMP		Suth-1995		D-model	
	n	B_f	A_f	B_f	A_f	B_f	A_f	B_f	A_f
All Media	75	**0.79**	1.66	0.63	1.63	0.71	1.55	0.77	**1.53**
#1-TSB	8/4	0.43	2.33	0.42	2.40	**0.57**	**1.76**	0.55	1.82
#2-NB	19/16	0.61	1.65	0.48	2.07	**0.74**	**1.44**	0.66	1.59
#3-NB	9	0.76	1.31	0.61	1.63	**1.06**	**1.10**	1.09	1.14
#4-'broth'	7	0.81	1.23	0.69	1.45	0.97	**1.13**	**1.01**	1.18
#5-TSB	9/8	**0.60**	**1.65**	0.57	1.77	0.28	3.60	0.50	2.02
#6-BHI	23	1.38	1.77	0.86	**1.27**	0.88	1.38	**0.98**	1.47
All Meat	27	**0.89**	**1.32**	0.62	1.61	0.61	1.73	0.63	1.64
#7- beef	6/5	**1.07**	1.48	0.67	1.50	0.84	1.41	0.85	**1.32**
#8-beef	4	**0.70**	**1.43**	0.51	1.97	0.41	2.47	0.45	2.20
#9-mutton	8/7	**1.04**	**1.09**	0.88	1.14	0.79	1.29	0.78	1.34
#10-beef	9	**0.77**	**1.40**	0.50	2.00	0.46	2.17	0.50	1.99
All Media and Meat	102	**0.82**	**1.56**	0.63	1.63	0.68	1.59	0.73	**1.56**

#1=Doyle and Schoeni (1984); #2=Ingraham (1958); #3=Jennison(1935); #4=Barber (1908); #5=Glass *et al.* (1992); #6=Buchanan and Klawitter (1992); #7= Gill and DeLacy (1991); #8=Gill and Newton (1980); #9=Smith (1985) and #10=Grau (1983). BHI=Brain Heart Infusion Broth, NB=Nutrient Broth and TSB=Tryptone Soya Broth.

4.3.3 Model evaluation: industry

The results from the carcass chilling verification study are analysed and discussed in detail by Ross (1999). They are summarised in S. 4.4.2.

4.4 DISCUSSION

4.4.1 Evaluation of a new predictive model for *E. coli* growth

As yet, no criteria have been established by which a model can be said to be validated (Ross, 1996). However, it is generally accepted that the usefulness of a predictive model can be assessed by comparing predicted responses to observed responses. The observed response can come from a variety of sources: laboratory studies in media or foods, the published literature or industry trials. The ability of a predictive model to perform in

'real life' situations has already been emphasised as an important part of the model evaluation process. However, difficulties often arise in performing evaluation studies in the 'real life' environment, i.e. industry (*see* S. 4.1.1.3). Consequently, much of the data generated for model evaluation purposes is derived from laboratory studies or from literature data.

A predictive square-root type model for *E. coli*, Eqn. (18), was developed and its performance compared to other published predictive models for *E. coli* growth. Data for the comparisons were derived from laboratory studies in complex laboratory media, inoculation studies in ground beef and from the published literature.

4.4.1.1 Methodological considerations for laboratory performance evaluations

Prior to commencement of the laboratory inoculation studies in ground beef, the potential problems in the methodology were addressed. Media were assessed for their suitability to recover potentially injured cells and a method for enumerating target cells in the presence of naturally contaminating microbiota was selected.

While ground beef is not truly representative of the structure of a meat carcass it was considered adequate for laboratory performance evaluation purposes as it shares the same physiochemical properties (i.e. pH, a_w and chemical composition). Ground beef has been used as a substrate for evaluation of microbial growth by several authors (Mackey and Kerridge, 1988; Abdul-Raouf *et al.*, 1993; Kamperman, 1994; Walls and Scott, 1996; Palumbo *et al.*, 1997). For this study, ground beef was selected over products more representative of carcass surfaces, for example beef slices or roast portions, because preparation of an axenic product was simple and allowed homogeneous distribution of inoculated bacteria. The effect of naturally contaminating microbiota could also be examined easily by addition of a small proportion of normal retail product to the axenic ground beef. This introduced normal microbiota in sufficiently low numbers to allow growth of target organisms, i.e. to prevent the Jameson effect (S. 4.1.1.4.2). In this study, it was noted that the presence of

naturally contaminating microbiota affected the growth of *E. coli* at low temperatures, ≤12°C, in comparison to growth in axenic ground beef. The growth rate appeared lower in the retail/axenic mixture than its axenic counterpart at 12°C, and at 10°C growth was not sustained. These results suggest that for a laboratory performance evaluation to be effective, the presence of naturally contaminating biota should be taken into consideration.

A commercial dry-film plate was assessed for its suitability to recover potentially injured cells. 3M™ Petrifilm™ *E. coli* Count plates were suitable for the recovery of unstressed cells but too selective for recovery of cells stressed by low a_w and low temperature. This has implications for the meat industry as PECC is often employed in abattoirs for routine assessments of meat and meat products (Anon., 1998). Conditions at the carcass surface are likely to sublethally injure cells, thus PECC may provide inaccurate estimates of *E. coli* and coliform numbers, i.e. lower numbers than actually present.

As the effect of naturally occurring microbiota on the growth characteristics of *E. coli* in ground beef was of interest, a method was required to allow easy detection of the target *E. coli* strain. A streptomycin resistant *E. coli* strain was isolated to fulfill this need. Conflicting reports in the literature, *see* S. 4.1.1.4.3, concerning differences in growth rate between parent and mutant strains led to investigation of the growth rate of the mutant strain used in the inoculation studies. The results indicated that, for temperature, the growth rate of the antibiotic resistant strain is slightly slower than that of the parent strain. To test this observation statistically, linear regression analysis was undertaken to determine if the T_{min} estimates were different. The streptomycin resistant *E. coli* M23 had a T_{min} close to that predicted by most models, approximately 4°C. Interestingly, the T_{min} for the parent strain was lower at around 1°C. This probably serves to highlight that the data set is too small, i.e. 4 points, to draw any significant conclusions and that the experiment should be repeated with sufficient data to determine T_{min} with confidence. None-the-less, if an antibiotic resistant strain differs significantly in growth from the

parent strain it may be necessary to apply a calibration factor to growth rate estimates when using that strain to evaluate model performance.

4.4.1.2 Assessment of model performance

Graphical methods were used initially to assess model performance, however the basis of the evaluation of Eqn. (18) was calculation of the bias and accuracy factors. Before the results of the evaluations can be discussed it is first helpful to consider the practical significance and interpretation of the bias and accuracy factors.

4.4.1.2.1 Critical values for the bias and accuracy factors

Although there are no published critical values for the bias and accuracy factors that allow interpretation of these performance indices for model evaluations, some proposed values are "in press" (Dalgaard, (*in press*).

Bias factor

To reiterate, a bias factor of 1 indicates no systematic over- or under-prediction between observed and predicted generation times. The significance of the magnitude of the bias factor depends on the amount of growth that occurs, and is therefore dependent on whether the model being assessed is a model for spoilage or growth of pathogenic organisms. For example, if the bias factor were 0.9 and considering that the error in any viable count method is approximately ± 0.3 log cfu (Jarvis, 1989), differences between observed and predicted growth would not be measurable unless more than three log cycles of growth had occurred (i.e. 10% of 3 = 0.3). From a shelf-life perspective such an increase may be acceptable. However, for proliferation of pathogens with low infective doses, such an increase would be unacceptable. Thus, the bias factor must be interpreted in a manner consistent with the situation in which the predictive model is likely to be utilised.

Dalgaard (*in press*) suggests a B_f in the range 0.75 to 1.25 indicates a successful evaluation of seafood spoilage models. Ross *et al.* (1999) proposes the following interpretation of the bias factor when used for model performance evaluations involving pathogens:

- 0.90 - 1.05 is considered good
- 0.70 - 0.90 or 1.06 - 1.15 is considered acceptable
- <0.70 or >1.15 is considered unacceptable

Ross *et al.* (1999) considered that less tolerance should also be allowed for bias factors >1 as they would lead to under-predictions of pathogen growth and are thus 'fail-dangerous' predictions.

Accuracy factor

If the bias factor is close to 1, the accuracy factor provides an indication of the extent of variability that can be expected in responses. If the bias factor differs significantly from 1 then the accuracy factor reflects this systematic deviation. For example, an accuracy factor of 1.5 indicates approximately half of the observed generation times are within 50% of the predicted generation times.

Determining acceptable levels for the accuracy factor is problematic. Normal experimental error and biological variability contribute to variations in data derived from "internal" experiments, i.e. from experiments conducted by the researcher undertaking the evaluation, and are reflected in the accuracy factor. It is likely that this variability will be significantly larger when using externally derived data for comparisons; i.e. data other than that used to generate the model and in particular, data independent of the investigator's. Ross (1996) observed that model accuracy decreased as the degree of experimental control is reduced. Data for growth derived by that author from the simplest and most homogenous system, i.e. laboratory media, had the best model accuracy, 1.20. Using non-sterile, non-homogenous foods as the growth medium and using the modelled strain as the test organism under well controlled laboratory conditions, the model accuracy decreased to 1.26 and reflected the reduction in experimental control. An evaluation using independent food-based data from the literature decreased model accuracy even further to 1.53. This is presumably due to errors and variability introduced by: estimations of generation time from growth curves, lack of uniformity in methods of growth rate determination and lack of relevant information on some parameters such as pH, temperature, a_w and strain variability.

Model performance reported in this thesis and those of Neumeyer *et al.* (1997) support the observations of Ross (1996). For the growth model for *E. coli* presented in this thesis, Eqn. (18), model accuracy was 1.15 in growth experiments conducted by the candidate in complex laboratory media (*see* A. 9.4.4) and in inoculated, non-sterile ground beef (*see* A. 9.4.5). Model accuracy for the 'unedited' literature data set, i.e. all the literature data, was poorer, $A_f = 1.42$. Similarly, in an evaluation of a growth model for *Pseudomonas putida* 1442, Neumeyer *et al.* (1997) reported model accuracy of $A_f = 1.23$ in laboratory media and 1.10 in homogenous liquid foods (milk and milk products), and 1.30 for a comparison to an unedited literature data set. It should be noted that the poorer model performance for the liquid media in the case of Neumeyer *et al.* (1997) was attributable to inter-strain differences; the majority of the inoculation studies in the milk and milk products involved the strain used to make the model, whereas the majority of the media determinations were for a 5-strain cocktail of slower-growing strains (this is reflected in the low bias factor).

The error in growth rate estimates under controlled laboratory conditions is estimated to be around 10% per independent variable (Ross *et al.*, 1999). Those authors proposed that as a 'rule of thumb', each environmental variable in a model can be expected to add a similar amount of relative error. Thus, an acceptable accuracy factor can be determined by considering the effect of the *number* of environmental parameters in a kinetic model. For example, the best performance that might be expected from a kinetic model encompassing the effect of temperature, pH and a_w on growth rate, is ~30%, or an accuracy factor of 1.3.

The critical values described above for the bias and accuracy factors enable objective performance evaluation of predictive models.

4.4.1.2.2 Model performance: graphical methods

While the bias and accuracy factors allow an objective summary of the performance of predictive models, they are imperfect because systematic

deviations in predictions between observed and predicted responses may be obscured (Ross, 1996). Thus, graphical methods for comparison of observed versus predicted growth are also important.

The graphical comparisons presented in Fig. 4.7 and the residuals plot (Fig. 4.9) revealed that, overall, Eqn. (18) describes the data well. For the 'edited' literature data set there is little evidence of any systematic deviation in predictions. The model appears to be conservative under conditions which lead to slow growth (i.e. longer generation times), as evidenced by the wider degree of scatter on the right of the residuals plot and the greater number of points below the line of equivalence in Fig. 4.7. This behaviour is, however, consistent with other reports in the literature (McClure *et al.*, 1994; Sutherland *et al.*, 1995; Miles *et al.*, 1997; Neumeyer *et al.*, 1997; Salter, 1998; Augustin and Carlier, 2000). These outlying points are usually generated under unfavourable conditions for growth. More points fall below the line of equivalence in Fig. 4.7 and this is reflected in the bias factor calculated for that data set which is less than one ($B_f = 0.92$).

For the 'meat' data set, which includes results from the laboratory inoculation studies in ground beef described in this thesis, Eqn. (18) describes the data well with most of the points lying close to the line of equivalence in Fig. 4.8. This is also reflected in a bias factor close to one ($B_f = 0.97$). However, for the 'other foods' category it is evident that Eqn. (18) describes the data 'less well'. The majority of predictions are much faster than the observed growth, thus the model is too conservative. This is reflected in the poor bias factor ($B_f = 0.58$).

The results from the classical graphing methods support the validity of the calculated bias factors. The limits proposed by Ross *et al.* (1999) can be used to evaluate the performance of Eqn. (18).

4.4.1.2.3 Model performance: bias and accuracy factors

Eqn. (18) performs well overall in the laboratory performance evaluation with a bias factor of 1.04 and an accuracy factor of 1.22 (Table 4.4). Closer scrutiny of the data reveals that while the accuracy factors are similar,

there is a difference in model predictions between the axenic and retail mix ground beef; the high bias factor of the former, $B_f = 1.20$, is balanced by the low bias factor of the latter, $B_f = 0.87$. The same pattern was observed when other models were compared to the laboratory generated data. Of the other models assessed for all the laboratory determinations, the Ross (1993) model was in the 'good' category for bias and had an accuracy factor similar to that of Eqn. (18), $A_f = 1.19$. Both PMP and FMM were in the acceptable category, $B_f = 0.85$ and 1.08 respectively, and had accuracy factors the same as that of the Ross (1993) model. Although Eqn. (18) performs well overall and is comparable to the three other models for *E. coli* growth, it should be noted that only a small number of growth data, $n = 7$, and one variable, temperature, were compared. An assessment of model performance against a larger, multivariate data set is a more rigorous and useful means of model performance evaluation.

Using growth data from the literature, Eqn. (18) performs well in almost all of the literature categories assessed (Table 4.5). From the literature performance evaluation, the bias factors for the 'edited' ($B_f = 0.92$), 'media', ($B_f = 0.93$) and 'meat' ($B_f = 0.97$) data sets all fall into the best level proposed by Ross *et al.* (1999), i.e. 0.90 to 1.05. Additionally, as the bias factors are <1, the model can be considered 'fail-safe'. Eqn. (18) is a four parameter square-root type model, therefore according to Ross *et al.* (1999) the best performance that can be expected from Eqn. (18) is an accuracy factor of ≤1.4. The accuracy factors of the 'edited' (A_f=1.29), 'media' (A_f=1.28) and 'meat' (A_f=1.26) data sets are all <1.4, indicating that Eqn. (18) has an acceptable level of accuracy. Although Eqn. (18) performed less well for the 'food' data set, $B_f = 0.86$ and $A_f = 1.36$, both of the performance indices are close to the levels considered to indicate a 'good' level of performance. This was also true for the unedited literature data set (B_f=0.85 and A_f =1.42).

The poorest level of performance for Eqn. (18) occurred for the 'other foods' category ($B_f = 0.62$ and $A_f = 1.71$). It was noted previously that the majority of foods in this category are milk products, which account for 41

out of the 48 growth observations. Closer scrutiny of the individual data sets (*see* A. 9.4.5), and the results presented in Figures 4.8 and 4.9, indicate that the low bias and high accuracy factors are observed consistently, as opposed to over- and under-predictions cancelling each other out. It is possible that the absence of aeration in such products may affect growth rate, and therefore be responsible for the disparity observed between the generation times reported and those predicted by a model constructed in well aerated laboratory media. This suggestion is supported by the pseudomonad model validation work of Neumeyer *et al.* (1997). They observed poorer bias and accuracy factors in minimally aerated low fat milk in comparison to well aerated product. Additionally, Kauppi *et al.* (1996) reported similar growth rates of *E. coli* in static cultures of Tryptone Soya Broth, BHI and milk, suggesting that it is not the milk itself affecting growth rate.

Eqn. (18) was critically evaluated against a large literature data set using graphical methods and the performance indices of bias and accuracy factors. It was demonstrated to perform well for a variety of data groups. To complete the assessment of Eqn. (18), a comparison was undertaken to assess model performance. Bias and accuracy factors were used to compare predictions from two other models for *E. coli* growth, PMP and *p*FMM-*Ec*, to the entire literature data set. Additionally, a sub-set of the literature data presented in Sutherland *et al.* (1995) contained predictions from two other polynomial-type models, based on either a modified Gompertz model or the Baranyi D-model. Bias and accuracy factors were used to compare the validity of predictions from the two models presented in Sutherland *et al.* (1995), and for Eqn. (18) and PMP to the data presented in Sutherland *et al.* (1995).

For the entire literature data set Eqn. (18) consistently performed better, i.e. had better bias and lower accuracy factor, than PMP or *p*FMM-*Ec* for the majority of the data sets used in the comparison (Table 4.5). Despite that PMP predictions were often close to those of Eqn. (18), Eqn. (18) clearly outperformed this model for meat. It should be noted that due to differences in the model limits, Eqn. (18) was compared to an average of 24% more data than PMP.

The exceptions to the good performance of Eqn. (18) were the 'unedited' and 'other foods' data sets. *p*FMM-*Ec* appeared to perform extremely well for the unedited data set, with a good bias factor of 1.07. However, it should be noted that the unedited data set contained many predictions outside the model limits for *p*FMM-*Ec*. Eqn. (18) was compared to approximately twice as much data. Closer scrutiny of the data sub-sets reveals that *p*FMM-*Ec* performs poorly in the 'media' and 'food' data sets. As these two categories comprise the bulk of the unedited data set, it is likely that the over-prediction in media is cancelled out by the under-prediction in the foods. Thus for comparative purposes, the 'edited' data set is more appropriate, and although *p*FMM-*Ec* had an acceptable bias factor according to the limits of Ross *et al.* (1999), Eqn. (18) performed better. For the 'other foods' category, although *p*FMM-*Ec* had better bias and accuracy factors than Eqn. (18), its performance was still considered poor. As no model performed well for this category, it would appear that the applicability of any of the three models for *E. coli* growth in these products is questionable.

As *p*FMM-*Ec* predicts the effects of three variables, this model is expected to have an accuracy of around 1.3. The accuracy factors calculated for most of the categories for *p*FMM-*Ec* were higher than 1.3, but covered a narrow range (1.46 to 1.53). This suggests that no matter how the data are subdivided or regrouped the accuracy factor will not improve.

The performance of Eqn. (18) was also assessed against subsets of the data divided on the basis of generation time (Table 4.6). The generation times were divided into <1 hour, 1 to 5 hour, and >5 hour subsets to characterise the 'stressfulness' of the environment. Thus, close-to-optimal environmental conditions are characterised by short generation times and conditions closer to the limits for growth are characterised by long generation times. The 1 to 5 hour category is intermediate between the two. Eqn. (18) consistently performed best at the fast and intermediate generation times. This supports the results from the graphical comparisons, in which it was observed that the predictions of Eqn. (18) were less reliable at slower generation times. This result is not unexpected

due to the high degree of variability in generation time estimates under conditions where growth is slow (McMeekin *et al.*, 1993). Once again the notable exception was the 'other foods' category. The anomaly of this data set has been discussed previously.

In comparison to Eqn. (18), PMP performed best for no generation time category although predictions were often similar between the two models , e.g. <5 hours for media. As for Eqn. (18), the predictions by PMP were better than those of *p*FMM-*Ec.* The best performance for PMP was observed at rapid generation times, i.e. <1 hour, and the performance indices were often close to those calculated for Eqn. (18). However, as generation time increased, predictions became consistently more unreliable, suggesting this model should only be used with confidence under conditions where rapid bacterial growth is expected.

*p*FMM-*Ec* out-performed both Eqn. (18) and PMP at generation times >5 hours. While bias factors were good for most data categories, accuracy was poor, suggesting predictions are potentially unreliable.

When compared to the subset of the literature data that were presented in Sutherland *et al.* (1995), Eqn. (18) performed best overall and for the 'meat'-specific category (Table 4.7). Inspection of the 'meat' data sets reveals that this performance was consistent, with best bias and accuracy factors observed for practically all of the individual data sets. For the media data, although Eqn. (18) had the best bias factor overall, ($Bf = 0.79$), and the Baranyi D-model the best accuracy, ($Af = 1.53$), the models generally performed poorly and exhibited similar bias and accuracy factors. Examination of the results for the individual data sets reveals that no one model consistently performed best for the media data. PMP consistently performed the worst in all categories.

The consistently good performance of Eqn. (18) for the 'meat' data presented in Sutherland *et al.* (1995) perhaps stems from the inclusion of the lactic acid term in the model, a parameter that is not explicitly included in the other models used in the comparisons. This hypothesis was tested by removing the lactic acid terms from Eqn. (18) and re-calculating the

bias and accuracy factors for all the meat data from the literature collated by the candidate. With the lactic acid terms included, bias and accuracy factors for all the meat data from the literature, i.e. not limited to that presented in Sutherland *et al.* (1995), were 0.97 and 1.26 respectively (*see* Table 4.5). When the lactic acid terms were removed from Eqn. (18) the bias and accuracy factors were 0.78 and 1.39. Thus the hypothesis that inclusion of the lactic acid terms in Eqn. (18) is responsible for the improved predictions for growth of *E. coli* in meat products is supported.

Comparison of the performance of Eqn. (18) against the three other models also highlighted the influence of fidelity of the data used in the comparison. Under circumstances where a number of models predict poorly it may indicate a deficiency in the literature data itself. Literature data may lack completeness in providing information on experimental design and methods, or insufficient data may have been generated for growth calculations. Consistently poor model predictions may also reveal inter-strain differences.

The use of correction factors for comparing data derived by different experimental techniques and analytical methods has been highlighted as an important consideration in model evaluations (S. 3.1.3). Eqn. (18) gave poor predictions for the data of Doyle and Schoeni (1984) and Glass *et al.* (1992) in tryptone soya broth (Table 4.7). In both of those publications, no information was provided on how growth rates were calculated. It is probable that the poor predictive ability of Eqn. (18) in this case was due to a deficiency in the data rather than a problem inherent in the model. This is supported by the observation that all four models gave poor predictions for these data; bias factors were all <0.60 and accuracy factors >1.65.

Eqn. (18) also predicted poorly for the data of Ingraham (1958) in nutrient broth using the *E. coli* strain K12 with B_f = 0.61 and A_f =1.65 (Table 4.7). The only other data set found for *E. coli* K12 is presented in Gill and Phillips (1985). When compared to their data for studies in unmodified BHI broth, Eqn. (18) has a bias of 0.65 and an accuracy of 1.53 (*see* A. 9.4.4). Given the similarity in the values of the performance indices for the

Ingraham (1958) and Gill and Phillips (1985) data, it is likely that Eqn. (18) gave poor predictions due to differences in growth characteristics between the *E. coli* K12 strain used in those studies and the strains modelled by Eqn. (18). The other models assessed also predicted poorly for these data (bias factors were all <0.74 and accuracy factors >1.44), and it is likely that strains other than *E. coli* K12 were used to produce those models. Poor model predictions due to inter-strain differences are not unexpected as 'predictive models are generally produced using fast growing strains so that predictions are guaranteed to be 'fail-safe' (McMeekin *et al.*, 1993).

4.4.1.2.4 Usefulness of literature data

Literature data has been used successfully to evaluate performance of a predictive model in this thesis. McClure *et al.* (1994), Ross (1996), Miles *et al.* (1997) and Neumeyer *et al.* (1997) consider there to be many problems inherent in using literature data in model performance evaluations. Concerns centre mainly on deficiencies in the literature data and decreased experimental control which is reflected in a higher accuracy factor. While the results from the literature performance evaluation conducted in this thesis support those authors' concerns, the role of a literature performance evaluation should not be undervalued. A search of the literature yielded 1217 growth rate data, which was edited to 1003, in a period of approximately 8 weeks. A similar time period was required to perform the laboratory inoculation studies in ground beef (S. 4.2.1.2) which yielded 7 growth rate data. Indeed, the role of literature data in predictive model evaluations can be optimised by implementation of assessment criteria, such as those described in S. 4.2.2.1, to improve the quality of the literature data used in evaluations. Such editing of the literature data set allows model performance to be assessed more fairly, i.e. the model's performance is not prejudiced by unrepresentative data. For Eqn. (18), scrutiny of the literature data resulted in an 'edited' data set where $Bf = 0.92$ and $Af = 1.29$. This is an improvement on the values for the 'unedited' data set, where the bias and accuracy factors were 0.85 and 1.42 respectively.

4.4.1.3 Summary

There are many potential problems in estimating growth data in non-homogenous and non-sterile foods. In this study, steps taken to minimise these problems included selection of a recovery medium for potentially injured cells and production of an antibiotic resistant strain to allow enumeration in the presence of natural microbiota. The potential difference in growth kinetics of a parent and mutant strain were considered, however, this did not invalidate the use of the antibiotic resistant strain in the laboratory performance evaluations of Eqn. (18).

The square-root type model for *E. coli*, Eqn. (18), underwent rigorous performance evaluation against a variety of data. These data came from inoculation studies in ground beef, laboratory studies in complex media and from the literature. Using critical values for bias and accuracy, Eqn. (18) performed well against food-based and media-based data. In a detailed comparison to other predictive modes for *E. coli* growth, PMP and pFMM-*Ec*, Eqn. (18) generally performed better. Similarly, when Eqn. (18) was compared to three other models for a smaller literature data set for growth in media and meat it performed best overall and exceptionally well in the 'meat' category. This was attributed to inclusion of the lactic acid terms in Eqn. (18). Poor performance against the media data was not confined to Eqn. (18); all models generally performed poorly and it is possible that inter-strain differences were a contributing factor.

A notable exception to the good performance of Eqn. (18), was for literature data from the 'other foods' category which comprised milk and milk-based products. Lack of aeration is proposed as a possible explanation. That PMP and *p*FMM-*Ec* also showed poor performance for this category indicates that poor predictive ability of Eqn. (18) for these foods is not a problem unique to the model. Eqn. (18) consistently predicted better at faster generation times, i.e. GT ≤5 hours. This is a significant observation as most users of predictive models will be interested in predicting the consequences of conditions that lead to slow growth rates.

The performance evaluation of Eqn. (18) has highlighted some of the difficulties and subtleties of the model evaluation process. Unless data are collected under well controlled conditions, a degree of variability in data can be expected due to normal experimental error and biological variability, and this variability is likely to be larger for data obtained from the literature. The performance indices of bias and accuracy factors are useful tools in the model evaluation process, however they do not generate absolute measures of performance: the values of the performance indices will be specific to the data sets used in the evaluation and care should be taken in their interpretation. The value of the data presented in the literature should not be underestimated, however an awareness of its limitations is essential.

4.4.2 Carcass chilling verification study

It should be emphasised that the candidate was responsible for model development only. However, the results of the carcass chilling study are discussed because they provided one of the first opportunities for evaluation of predictive microbiology under normal commercial operations in an Australian abattoir.

The results from the carcass chilling verification study were analysed and discussed by Ross (1999). He reported that the model over-predicted the extent of growth on carcasses during chilling. However, when the substantial lag period after inoculation of the test organism on to the carcass was taken into account, i.e. around 4 to 5 RLT units, there was good agreement between the model's prediction and the observed growth of *K. oxytoca* (*see* S. 5.1.3 for more on RLT). The lag period information in that study was derived from the studies reported in this thesis for osmotic and temperature shifts (*see* Chapter 5), and from a literature survey on RLT distributions. The results presented in Ross (1999) demonstrate a high degree of variability in the responses of *K. oxytoca* at individual sites on individual carcasses. Despite this variation, he concluded that "models *can* perform accurately in commercial situations to predict the average, or 'most-likely' effect of carcass cooling processes, but that due to variability in the organisms, sites of contamination on the carcass, carcass position within the chiller etc., the

outcome on an individual carcass cannot be well predicted. That is, predictive models can be used to assess the performance of a *process*. It will be more difficult to apply predictive microbiology to assess the microbiological status of individual units of product because of the high degree of variability" (Ross, 1999).

5. ENVIRONMENTAL SHIFTS AND LAG PHASE DURATION

5.1 INTRODUCTION

5.1.1 Modelling the lag phase

During the lag phase cells adjust to their new environment by inducing or repressing enzyme synthesis and activity, initiating chromosome and plasmid replication and, in the case of spores, differentiating into vegetative cells (Montville, 1997). The resolution of lag is not a synchronous, abrupt event, rather a gradual transition as more and more cells in the population commence growth (McMeekin *et al.*, 1993). The population lag should not be considered as reflecting the average lag of individual cells, rather the distribution of lag times of individual cells is a more appropriate measure (Baranyi, 1998).

Lag phase duration is usually calculated by extrapolating the curve of the exponential phase to the level of the population at the time of inoculation (Ingraham *et al.*, 1983). Response surface models generated from stationary phase cultures describe the entire growth curve, and thus provide lag time predictions. Square-root type models, which are employed in this thesis, describe exponential growth rate but can be equally well used to model the reciprocal of lag time in response to temperature if stationary phase cultures are used. Chandler (1988) investigated the effect of temperature on the growth rate and lag rate, (the reciprocal of lag time), of a pseudomonad isolated from milk. Using square root type models he determined the T_{min} values to be 267.9 and 267.3K respectively. Similarly, McMeekin *et al.* (1987) reworked the growth rate and lag data of Smith (1985) for *E. coli* and determined the T_{min} values to be 275.8 and 274.9K respectively. Square-root type models for the reciprocal of lag time are yet to be investigated for other environmental parameters.

5.1.2 Environmental effects on the lag phase

Generation times, GT, of a species are reproducible in a specified environment, but the lag time is dependent on the physiological history of the cell. The duration of the lag phase is affected by factors such as the identity and phenotype of the bacterium (Buchanan and Cygnarowicz, 1990), inoculum size (Baranyi and Roberts, 1994), the physiological history of the population (McMeekin *et al.*, 1993) and by changes in the physiochemical environment such as temperature (Zwietering *et al.*, 1994), pH, a_w and nutrient availability (Buchanan and Cygnarowicz, 1990). Larger inocula usually have a shorter lag time (Montville, 1997). It is hypothesised that the lag phase is shorter if the inoculum is more metabolically active, i.e. exponential phase, than if activity is low, i.e. stationary phase (Baranyi and Roberts, 1994). The lag phase can be modelled, along with maximum specific growth rate, as a function of environmental factors such as temperature, pH and a_w (Baranyi *et al.*, 1995). However, predictions can only be made reliably if the physiological history of the cells is known. Typically it is not and often a worst-case strategy for lag times is adopted. This assumes that none of the microorganisms introduced or present in the food have a lag time before (re)commencing growth. It is likely that this approach could lead to over-prediction of the severity of the risk from pathogens, or of the extent of spoilage, and wastage of acceptable product could result.

While studying the effect of temperature shifts and fluctuations on growth rate, several authors have noted effects on the lag phase (Walker *et al.*, 1990; Buchanan and Klawitter, 1991; Fu *et al.*, 1991; Blackburn and Davies, 1994b; Baranyi and Pin, 1999; Membre *et al*, 1999). With regard to temperature, it has often been observed that lag is inversely proportional to the maximum specific growth rate (Smith, 1985; Mackey and Kerridge, 1988; McMeekin *et al.*, 1993; Baranyi and Roberts, 1994). Shaw *et al.* (1971) states from his observations that "as the temperature to which the culture is transferred is decreased, the length of time before growth recommences is increased". Chohji and Sawada (1983), when examining the effect of temperature shifts at lower nutrient concentrations observed that the duration of the lag phase in temperature upshifts at low glucose concentrations was primarily determined by the post-shift temperature.

If the incubation temperature of an exponentially growing culture is suddenly shifted within the normal (Arrhenius) temperature range, exponential growth continues at a rate characteristic of the new temperature (Neidhardt *et al.*, 1990). Rapid attainment of the maximum specific growth rate for the post shift temperature has been observed (Ng *et al.*, 1962; Shaw, 1967). However, if temperature shifts are made between the normal physiological range and either the high or low range, growth proceeds at transitional rates before exponential growth at a rate characteristic of the new temperature begins. Transient growth periods are most marked in shifts to or from the low range (Neidhardt *et al.*, 1990). Ingraham and Marr (1996) observed that a shift from the normal to the low temperature range exhibited a complex transient growth phase consisting of a lag followed by a period of abnormally rapid growth before the characteristic steady-state rate ensued. Similarly, a shift from the low to normal temperature range resulted in an extended period of growth (for about 2.3 doublings) at a low rate before growth at the characteristic steady-state rate ensued. Other authors have observed transitional periods of growth following shifts to or from the low to the normal range (Ng *et al.*, 1962; Shaw, 1967; Fu *et al.*, 1991; Baranyi *et al.*, 1995; Gay *et al.*, 1996). Shaw (1971), whilst determining the minimum temperature for growth for *E. coli,* also observed a transitional growth phase when a shift was performed between low temperatures outside the normal range.

Although temperature history effects have been the focus of most research into lag phase behaviour, other environmental parameters such as pH, gaseous atmosphere, nutrient content and a_w contribute to the dynamic environment of microorganisms. When microorganisms are grown on either side of their optimum pH range, an increased lag phase results (Jay, 1986). For *L. monocytogenes,* Robinson *et al.* (1998) observed a threshold between pH 4.5 and 5.0, below which very long lags occurred. In their experiments pH was the sole variable. Buncic and Avery (1995) observed an effect of prior incubation pH on the lag phase of two strains of *Aeromonas hydrophila* at 5°C. The lag phase appeared shorter in general after pre-incubation at pH 6.0 than at pH 7.0, with growth rates assuming

that of the new pH as expected. Osmotic shock in the form of a step up in sucrose concentration induced lag in *S.* Typhimurium LT2 with growth rates those expected of the new sucrose concentration (Brocklehurst *et al.*, 1995). Cheroutre-Vialette *et al.* (1998) induced lag phases in exponentially growing cultures of *L. monocytogenes* with acid (acetic acid) and osmotic (NaCl and KCl) shocks. Robinson *et al.* (1998) reported an increase in lag time duration of *L. monocytogenes* in response to a NaCl mediated decrease in a_w.

5.1.3 The relative lag time concept

As stated above, it has been observed that lag is inversely proportional to the maximum specific growth rate with regard to temperature. It is hypothesised that lag time responds to environmental changes much like growth rate, i.e. as generation time is a measure of metabolic rate in a new environment, so too lag time relates to the harshness of the environment. Robinson *et al.* (1998) and Ross (1999) hypothesised that lag could be determined by two parameters: the *amount* of work to be done to adapt to a new environment and the *rate* at which that work can be done. Robinson *et al.* (1998) defined work as the various biosynthetic and homeostatic responses needed to prepare for growth in the new environment. However, Ross (*pers. comm.* 1999) considered that the effects of increasing homeostatic demands are more likely to be manifested as reduced growth rate. Thus, the *amount* of work to be done to resolve lag, involves only the additional biosynthetic demands to adjust to the new environment. The *rate* of work is dependent on the harshness of the new environment and the extent to which it limits metabolic rate or diverts metabolic activity to homeostatic processes.

The *amount* of work to be done is a hypothetical quantity and as such cannot be directly measured. However lag time can be measured and the *rate* at which the work can be done can be inferred from the generation time, or more specifically from the growth rate, the reciprocal of generation time. Consequently if a bacterial culture is introduced into a new environment, the ratio of the lag time divided by the generation time in that environment will be a measure of the *amount* of work to be done by the cell before growth is initiated (Ross, 1999). This ratio can be

considered as the relative lag time, RLT, or "generation time equivalents" and is used in this thesis to monitor the response of bacteria to abrupt changes in their extracellular environment.

RLT can be used to define a range, or distribution of lag times, so that lag times can be incorporated reliably into predictive models and risk assessments.

5.1.4 Importance of the lag phase to the meat industry

The lag phase is of significance to the meat industry as a means of preventing the growth of undesirable microorganisms and extending the shelf life of products. Cooling of meat after slaughtering is considered a critical element in the control of enteric pathogens and strict codes of practice must be followed. However, Smith (1985), when investigating the effects of temperature on the kinetics of bacterial growth on meat, posed the question "are the codes of practice imposed on abattoirs realistic?". His studies on lag times indicated that the 10°C temperature limit on boning rooms was too stringent and that it is possible to hold meat above this temperature for certain time periods. The benefits included decreased costs and increased worker comfort, without compromising the safety of the product.

A better understanding of the lag phase may:

- provide greater precision in defining growth limiting conditions, thus preventing microbial proliferation on carcasses,
- allow development of novel protocols to optimise current chilling practices and ensure the microbial safety of the meat carcass,
- enable processors to express/determine the validity of their carcass processing technique in comparison to others,
- provide new insights and offer a reliable way to include the effects of lag time within the context of predictive microbiology.

5.1.5 Objectives

In this chapter, the effects of abrupt shifts in temperature, pH and a_w on the lag phase duration and growth kinetics of *E. coli* and other foodborne

bacteria are examined. RLT is used to characterise these responses. The RLT concept is also explored to test the hypothesis that lag times can be understood in terms of the *amount* of work to be done in adjusting to a new environment and the *rate* at which that work is done.

5.2 MATERIALS AND METHODS

5.2.1 Osmotic shift Experiments

To investigate the potential to induce a lag phase in exponentially growing cultures, a_w shifts at 25.5 (±0.4)°C in complex laboratory media were applied to 6 foodborne bacterial strains. For the two *E. coli* strains and *K. oxytoca*, similar experiments were conducted in a minimal medium. The potential effect of inoculum a_w history on growth rates in a new osmotic environment was examined concurrently to the lag phase experiments for some strains. Growth was monitored turbidimetrically in all these experiments. An additional set of a_w downshift experiments were performed with one of the strains, *K. oxytoca*, where growth was monitored by viable count to allow comparison of the results to the turbidimetric data. For the lag phase experiments conducted in minimal media with *E. coli* SB1, the potential effect of inoculum a_w history on 'cell yield' was also recorded. 'Cell yield' was also recorded for a a_w downshift experiment for *K. oxytoca* in minimal media. The potentially injurious effects of abrupt shifts from a high a_w to a low a_w were investigated by monitoring growth on a selective medium in some of the viable count experiments for *K. oxytoca*.

The terminology employed in all osmotic shift experiments refers to extracellular a_w and should not be confused with intra-cellular osmotic concentration. A *downshift* refers to moving from a high a_w to a low a_w, and an *upshift* refers to moving from a low or intermediate a_w to a high a_w.

5.2.1.1 Osmotic shifts in complex laboratory media

OVERVIEW

A primary inoculum of stationary phase culture was prepared in the appropriate basal medium. Aliquots were removed and inoculated into

either basal medium and/or NaCl adjusted media to provide late exponential phase inocula at various a_w. The secondary inocula were then dispensed into a series of a_w modified broths in which growth was monitored turbidimetrically. Generation time and lag time estimates were calculated using non-linear regression for all strains except *S. aureus*. Linear regression analysis was required for this strain due to 'non-sigmoidal' growth curves. Relative lag times, i.e. the lag time divided by the generation time, and lag rate, the reciprocal of lag time, were calculated.

METHOD

Late exponential phase inocula of *E. coli* SB1, *E. coli* R31, *S. aureus* (ATCC 25923), *L. monocytogenes* (L5/22), *S.* Typhimurium M48 and *K. oxytoca* (NRRL B-199) were prepared according to S. 2.1.2.1, with variations on the a_w of the growth medium for some of the secondary inocula. For a_w downshifts, i.e. moving from a high a_w to a lower a_w, unadjusted basal media were used.

For a_w upshifts, i.e. moving from a low or intermediate a_w to a high a_w, the following broths were used for growth of the secondary inoculum: for *E. coli* SB1 and *E. coli* R31; NB with 35g.L^{-1} NaCl added (a_w 0.980) and NB with 65g.L^{-1} NaCl added (a_w 0.960), and for *K. oxytoca* , BHI with 85g.L^{-1} NaCl added (a_w 0.949) were prepared based on the tables of Chirife and Resnik (1984).

For the test broths, the following basal media and media with added NaCl were prepared. For *E. coli* SB1 and *E. coli* R31, NB (a_w 0.999) and NB with 85g.L^{-1} NaCl added (a_w 0.946); for *S. aureus*, NB (a_w 0.998) and NB with 185g.L^{-1} NaCl added (a_w 0.946); for *K. oxytoca*, BHI (a_w 0.996) and BHI with 90g.L^{-1} NaCl added (a_w 0.941); for *S.* Typhimurium, BHI (a_w 0.993) and BHI with 105g.L^{-1} NaCl added (a_w 0.935); and for *L. monocytogenes*, TSB-YE (a_w 0.996) and TSB-YE with 120g.L^{-1} NaCl added (a_w 0.920). These pairs of basal media were combined in various ratios to generate a series of broths at a range of a_w values (S. 2.1.4). For *E. coli* SB1 and *E. coli* R31, 13 different ratios were tested; 24 for *S. aureus*; and 20 each for *K. oxytoca*, *S.* Typhimurium and *L. monocytogenes*.

L-tubes were pre-warmed with shaking in TGI-1 set isothermally at 25.5 (± 0.4)°C.

A small volume of the secondary inoculum was aseptically dispensed into each of the L-tubes. In the case of *E. coli* SB1 and *E. coli* R31, 0.5mL of the secondary inoculum culture was dispensed. For *K. oxytoca*, *S.* Typhimurium, *S. aureus* and *L. monocytogenes*, 0.3mL was dispensed.

Late exponential phase cultures of *E. coli* SB1, *E. coli* R31 and *K. oxytoca* were subjected to a_w upshifts and downshifts. Late exponential phase cultures of *S. aureus*, *S.* Typhimurium and *L. monocytogenes* were subjected to a_w downshifts only.

Growth was monitored according to S. 2.1.3.1.1 and curves analysed using non-linear regression (S. 2.1.3.2) to estimate values for generation time and lag time. RLTs and lag rates were calculated.

5.2.1.2 Osmotic shifts in minimal media

OVERVIEW

The effect of inoculum history, a_w, on the lag phase duration, growth rate and 'cell yield' of two gram-negative foodborne bacteria in minimal media was determined. *E. coli* SB1 was subjected to a_w shifts from a high (0.999), low (0.960) and intermediate a_w (0.980). *K. oxytoca* was subjected to a a_w downshift from a_w 0.997 only.

A primary inoculum of stationary phase culture was prepared in the appropriate basal complex laboratory medium. Aliquots were removed and inoculated into either a basal complex laboratory medium and/or NaCl adjusted complex laboratory medium to provide exponential phase inocula at various a_w values. The secondary inocula were then dispensed into a series of a_w modified minimal media broths in which growth was monitored turbidimetrically. Generation time and lag time estimates were calculated using linear regression due to 'non-sigmoidal" growth curves. 'Cell yield' was calculated by measuring the reduced final optical density.

METHOD

For *E. coli* SB1, 1/4 strength NB prepared in a base of Minimal Media, 0.25NB-MM, was used as the test broth for the osmotic shift experiments. Experiments for *K. oxytoca* were undertaken in 1/5 strength NB prepared in a base of Minimal Media (0.20NB-MM). However, inoculum preparation was undertaken in complex laboratory media. Late exponential phase inocula of *E. coli* SB1 and *K. oxytoca* were prepared according to S. 2.1.2.1 with variations on the a_w of the growth medium for some of the secondary inocula. For a_w downshifts, i.e. moving from a high a_w to a lower a_w, the appropriate basal complex laboratory medium was used for growth of the secondary inoculum. For a_w upshifts, i.e. moving from a low or intermediate a_w to a high a_w, the following broths were used for growth of the secondary inoculum: for *E. coli* SB1; NB with 35g.L^{-1} NaCl added (a_w 0.980) and NB with 65g.L^{-1} NaCl added (a_w 0.960) and for *K. oxytoca*, basal BHI (a_w 0.996). It should be noted that the primary and secondary inocula were prepared in complex laboratory medium. In all other a_w shift experiments the secondary inocula were prepared in basal media analogous to that of the test broths. Inoculum preparation according to the protocol described in S. 2.1.2.1 was not possible in minimal media as the cultures did not reach a sufficient level of growth at low a_w to provide a suitable inoculum density, i.e. growth ceased before a level of 20%T was reached. To standardise the inoculum preparation to allow comparison to other a_w shift experiments, a complex laboratory medium was used. Due to the small volume of inoculum used, ≤ 0.5mL, nutrient carry-over from the secondary inoculum to the test broths was minimal and unlikely to interfere with 'cell yield' results.

For the test broths, the following basal media and media with added NaCl were prepared. For *E. coli* SB1 , 0.25NB-MM (a_w 0.997) and 0.25NB-MM with 85g.L^{-1} NaCl added (a_w 0.947), and for *K. oxytoca* , 0.20NB-MM (a_w 0.996) and 0.20NB-MM with 80g.L^{-1} NaCl added (a_w 0.944). These pairs of basal media were combined in various ratios (S. 2.1.4). For *E. coli* SB1, 13 different broths of various a_w were used for each osmotic shift experiment, and for *K. oxytoca*, 24 broths were used.

L-tubes were pre-warmed with shaking in TGI-1 set isothermally at 25.5 (± 0.4)°C.

A small volume of the secondary inoculum was aseptically dispensed into each of the L-tubes. In the case of *E. coli* SB1, 0.5mL of the secondary inoculum culture was dispensed. For *K. oxytoca* 0.3mL was dispensed.

Growth was monitored according to S. 2.1.3.1.1 and curves analysed using linear regression (S. 2.1.3.3) to estimate generation and lag times. RLTs and lag rate were calculated. 'Cell yield' was measured as the change in optical density, calculated by subtracting the final optical density from the initial optical density.

5.2.1.3 Osmotic shifts and RLTs estimated by viable count

OVERVIEW

The effect of a_w downshifts on the RLT of *K. oxytoca* was determined by viable count. Growth was monitored simultaneously by turbidimetry to provide data for a model calibration exercise detailed in S. 3.2.2.2.1. The potential for hyperosmotic shift mediated injury was investigated on MacConkey Agar, MAC, a selective medium containing bile salts.

A primary inoculum of stationary phase culture was prepared in basal BHI. Aliquots were removed and inoculated into fresh basal BHI to provide a late exponential phase inoculum. The secondary inoculum was diluted in PW then dispensed into a series of eight NaCl modified BHI broths at various a_w. Growth was monitored by turbidimetry and by viable count on non-selective PCA growth media for all conditions. An additional medium, selective MAC, was used for viable count determinations for broths of a_w 0.992, 0.976 and 0.960. Generation time and lag time estimates from the viable count data were calculated using linear regression. Generation time estimates for turbidimetric data were calculated using non-linear regression.

METHOD

A late exponential phase inoculum of *K. oxytoca* in BHI was prepared (S. 2.1.2.1). The a_w of the BHI broth was 0.998. The inoculum was serially diluted in PW to a concentration of ~10^{5-6} cfu.mL^{-1}.

BHI at a_w 0.992, 0.984, 0.976, 0.968, 0.960, 0.953, 0.949 and 0.945 were prepared by mixing basal BHI (a_w 0.996) and BHI with 90g.L^{-1} NaCl added (a_w 0.941) in ratios as described previously (S. 2.1.4). 15mL aliquots of media were aseptically dispensed into sterile L-tubes.

L-tubes were pre-warmed with shaking in TGI-1 set isothermally at 25.5 (± 0.4)°C.

0.5mL of the diluted inoculum was aseptically dispensed into each of the L-tubes to achieve a concentration of ~10^{3-4} cells.mL^{-1}.

Growth was monitored turbidimetrically and by viable count on PCA (S. 2.1.3.1.2). Viable counts were also determined on MAC for three of the conditions (a_w 0.992, 0.976 and 0.960). Plates were incubated for 24 hours at 35°C and colonies counted manually.

Values for lag and generation time for viable count data were estimated using linear regression (S. 2.1.3.3). RLTs and lag rate were calculated. For the turbidimetric data, estimates for generation time only were calculated using non-linear regression (S. 2.1.3.2).

5.2.1.4 Sublethal injury in exponentially growing *K. oxytoca*

OVERVIEW

Two experiments were undertaken to investigate the potential effects of media type and incubation conditions on the recovery of exponentially growing cells of *K. oxytoca* after abrupt a_w downshifts.

For both experiments, a primary inoculum in stationary phase was prepared in basal BHI. Aliquots were removed and inoculated into fresh BHI to provide a late exponential phase secondary inoculum. The secondary inoculum was diluted in PW for one experiment and in NaCl

modified PW for the other. The secondary inocula were added to BHI at a_w 0.962 and incubated with shaking at 25.3 (± 0.2°C).

Growth in each experiment was monitored by viable count on a variety of media incubated at 25°C for 40 to 48 hours. Media used included non-selective PCA, selective MAC and PCA with 50g.L^{-1} NaCl added, PCA-S, and an enhanced resuscitation medium, Brain Heart Infusion Agar with 0.1% pyruvic acid added, BHAP. Addition of 0.1% pyruvate to media was shown to enhance the recovery of cells sub-lethally injured by treatment with organic acids (Leyer and Johnson, 1992) and heat (Mackey and Derrick, 1982a). Brain Heart Infusion Agar, BHA, was selected as it is an extremely nutritious medium.

To examine the effect of incubation temperature, for the experiment using PW diluent a duplicate set of PCA and MAC were prepared and incubated at 35°C for 18 to 24 hours.

Generation time and lag time estimates were calculated using linear regression (S. 2.1.3.3).

METHOD

A late exponential phase inoculum of *K. oxytoca* was prepared (S. 2.1.2.1.). The inoculum was serially diluted in PW in the first experiment, and in 0.1% Peptone Water with 50g.L^{-1} NaCl added, PWS, for the second experiment to a concentration of ~10^{5-6} cfu.mL^{-1}.

For each experiment, a 250mL sidearm flask containing 150mL of BHI broth at a measured a_w of 0.962 (50g.L^{-1} NaCl) was equilibrated for 6 hours in a shaking waterbath at 25.0 (± 0.1°C). 0.5mL of the diluted inoculum was dispensed into the test broth to a final concentration of ~10^{3-4} cells.mL^{-1}.

Prior to inoculation into each test broth, a viable count of the diluted inoculum was determined by spread plating of 0.1mL aliquots of appropriate dilutions suspended in PW or PWS onto PCA, MAC and BHAP, and incubating plates at 25°C for 24 hours. Colonies were counted

manually and the concentration of the cells in the diluted inoculum was estimated. This estimate was used to calculate the concentration of cells in each of the test broths after addition of the diluted inoculum, i.e. the additional dilution upon inoculation into the broth is taken into account. These estimates were plotted as the viable count at time zero.

Immediately post inoculation and at regular time intervals, viable counts were performed by surface plating 0.1mL aliquots onto appropriate media.

For the experiment using PW diluent, growth was monitored by viable count on non-selective PCA, enhanced resuscitation BHAP and selective MAC growth media. Plates were incubated at 25°C for 40 to 48 hours, with a duplicate set of PCA and MAC incubated at 35°C for 18 to 24 hours.

For the experiment using PWS diluent, growth was monitored by viable count on non-selective PCA, enhanced resuscitation BHAP, PCA-NaCl and selective MAC growth media using PWS diluent. All plates were incubated at 25°C for 40 to 48 hours as the preliminary results from the first experiment in S. 5.3.1.2 indicated enhanced recovery of the injured cells on the agar media at this temperature/time combination.

Lag and generation times were estimated for the viable count data using linear regression (S 2.1.3.3).

5.2.2 Temperature Shift Experiments

To investigate the potential to induce a lag phase in exponentially growing cultures, temperature shifts were applied to 2 gram-negative foodborne bacteria. The potential effect of inoculum temperature history on growth rates at a different temperature was examined concurrently. For *E. coli*, temperature shifts were applied to two cultures incubated at temperatures outside the normal physiological range for growth of *E. coli* reported by Ingraham and Marr (1996). For *K. oxytoca*, temperature shifts were applied to a culture incubated at a temperature inside the normal physiological range for growth, 25°C. This temperature was estimated to fall within the normal physiological range for *K. oxytoca* as no information

on the normal physiological range for this strain was found in the published literature.

Generation and lag times were estimated using non-linear regression (S. 2.1.3.2). RLTs and lag rates were calculated.

5.2.2.1 Effect of temperature shifts on *E. coli* SB1.

OVERVIEW

A primary inoculum of *E. coli* SB1 in stationary phase was prepared at 25 (±0.1)°C. Aliquots were removed and inoculated into sterile liquid growth media and incubated at 10.0 (± 0.1) and 44.4 (± 0.1)°C to provide secondary inocula of late exponential phase cultures. The secondary inocula were dispensed into a series of 24 broths over a temperature range of 9.9 to 47.1°C. Growth was monitored turbidimetrically and growth curves analysed using non-linear regression.

METHOD

A primary inoculum of *E. coli* SB1 in NB was prepared according to S. 2.1.2.1. The secondary inoculum was prepared with a variation on the incubation temperature of the growth medium. For temperature upshift experiments the secondary inoculum was incubated at 10.0 (± 0.1)°C with shaking, and for temperature downshift experiments it was incubated at 44.4 (± 0.1)°C with shaking.

L-tubes were pre-warmed with shaking in TGI-2 set for a temperature range of 9.9 to 47.1°C. Each side of TGI-2 contains 24 ports, and all 48 ports were utilised.

0.5mL of each of the secondary inocula was aseptically dispensed into 24 L-tubes, i.e. one set each side of the TGI.

Growth was monitored turbidimetrically according to the method described in S. 2.1.3.1.1. Generation time and lag times were estimated using non-linear regression (S. 2.1.3.2). RLTs and lag rate were calculated.

5.2.2.2 Effect of temperature shifts on *K. oxytoca*

OVERVIEW

A primary inoculum of *K. oxytoca* in stationary phase was prepared at 25 (±0.1)°C. Aliquots were removed and inoculated into sterile liquid growth media and incubated at 25 (±0.1)°C to provide secondary inocula of late exponential phase cultures. The secondary inocula were dispensed into a series of 30 broths over a temperature range of 0.5 to 47.0°C. Growth was monitored turbidimetrically and growth curves analysed using non-linear regression.

METHOD

A late exponential phase inoculum of *K. oxytoca* in BHI was prepared at 25.0 (±0.1)°C (S. 2.1.2.1).

L-tubes were pre-warmed with shaking in TGI-3 set for a temperature range of 0.5 to 47.0°C. Each side of TGI-2 contains 30 ports, and one side only was utilised..

0.3mL of the late exponential phase inoculum was aseptically dispensed into each of the 30 L-tubes.

Growth was monitored turbidimetrically according to S. 2.1.3.1.1. Generation time and lag times were estimated using non-linear regression (S. 2.1.3.2). RLTs and lag rate were calculated.

5.2.3 pH Shift-Data Collation and Analysis

Raw turbidimetric growth data were collated from Presser (1996) and Presser *et al.* (1997). In summary, stationary phase cultures of *E. coli* M23, 36h at 37°C, were prepared in NB at pH 7.3. 0.5mL of culture was inoculated into a series of 14.5mL NB at various pH (modified by the addition of either NaOH or HCl) covering the range 3.87-8.11 and incubated isothermally at 21.0 (±1°C). Growth curves, 49 in total, were constructed by measuring per cent transmittance (%T) at 540nm with a digital spectrophotometer at 540nm (*see* S. 2.1.3.1.1). This raw growth data was then analysed by the candidate.

For growth curve analysis, % transmittance data was transformed to optical density using Eqn (6), and log OD plotted against time. Curves were analysed using non-linear regression (S. 2.1.3.2) to provide estimates for generation and lag time. RLTs were calculated.

5.2.4 Distributions of RLT

RLTs for all the lag and generation time data generated in the experiments described in this chapter were calculated. The data were collated in Excel spreadsheets (Microsoft 98) and the Histogram function used to sort the RLT data into categories according to the magnitude of the RLT. The data were then plotted as frequency distributions.

5.3 RESULTS

Universal scales for *x* and *y* axes were not employed for the following plots to facilitate clear descriptions of patterns of kinetic responses for each bacterial strain.

5.3.1 Osmotic shift

5.3.1.1 Effects on bacterial growth kinetics

As described in S. 5.2.1.1, a_w downshifts of exponential phase inocula grown in complex basal laboratory media were performed on *E. coli* R31, *E. coli* SB1, *K. oxytoca*, *S.* Typhimurium, *L. monocytogenes* and *S. aureus*. Upshifts of an inoculum in NaCl modified media at a a_w close to the a_w limit for growth were performed on the gram negative strains from the above list. For *E. coli*, an upshift from the middle of the growth permissive range for a_w was also performed (a_w 0.980). An additional set of shifts was performed for *E. coli* SB1 in minimal media, and a single downshift experiment for *K. oxytoca* in minimal medium.

Effect of immediate osmotic shifts on the growth rate of *E. coli* SB1, *E. coli* R31 and *K. oxytoca*

The growth rate of *K. oxytoca* and both strains of *E. coli* declined approximately linearly with decreasing a_w below the optimum a_w for growth, in both complex and minimal media. Due to curvature of the plot near the optimum a_w for growth, growth rates for most of the growth rate versus a_w plots were compared by linear regression analysis using SAS PROC.GLM of a restricted data set that corresponded to the most linear portion. For both *E. coli* strains in complex media, linear regression analysis was performed on data below a_w 0.990. For *E. coli* SB1 in minimal media, linear regression analysis was performed on data below a_w 0.975. For *K. oxytoca*, data below a_w 0.970 was used. Where the lines intercept the *x* axis is the theoretical minimum a_w (a_{wmin}) for growth. If inoculum history does not affect growth rate in a new osmotic environment, it is expected that this notional a_{wmin} value will be similar for each data set, i.e. within the resolution limits of the a_w meter (0.003 a_w units).

E. coli SB1-complex laboratory media

For *E. coli* SB1 in NB, a_w shifts were in the range of -0.049 to +0.037 a_w units, i.e. the difference between the initial and final a_w. Raw experimental data are presented in A. 9.4.9.

Growth rate *vs.* a_w plots for the three inocula prepared at different a_w are presented in Fig. 5.1 and exhibit similar, but not identical, growth rate responses to a_w.

For *E. coli* SB1, some scatter in the plots near the optimum a_w for growth was observed. Linear regression of the three plots in isolation and the data set as a whole for data below a_w 0.990, was undertaken using SAS PROC.GLM. The slopes of the regression lines were found not to be identical, however they were not significantly different (P=0.1214). The slope of each line was fixed to the common slope value to determine if the *x* axis intercepts were different. This analysis revealed that the intercepts were significantly different (P=0.0037). a_{wmin} values were calculated and are given in Table 5.1.

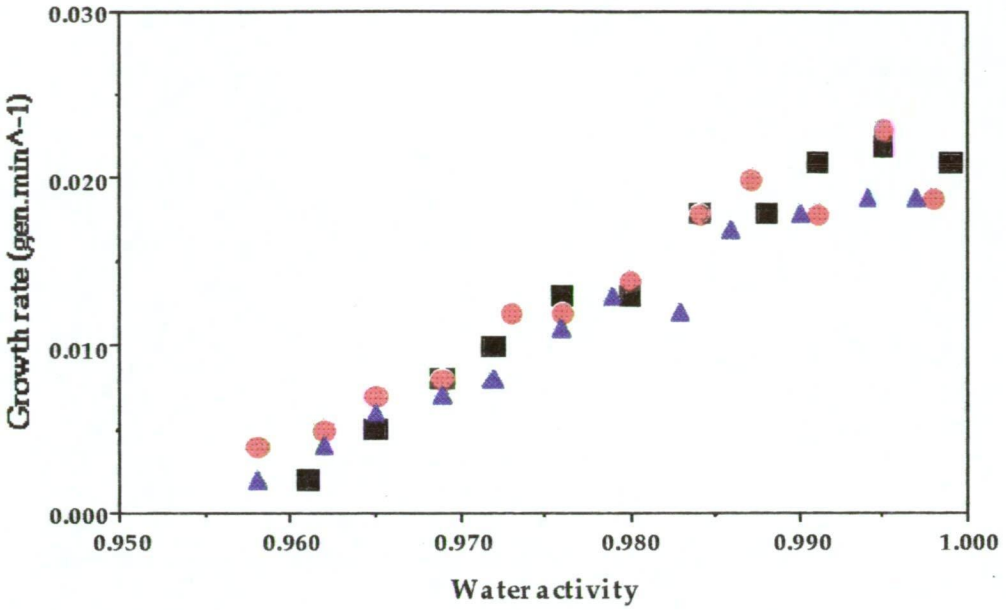

Figure 5.1: Effect of a_w shifts on the growth rate of *E. coli* SB1 in Nutrient Broth determined by turbidimetry, where: ■ = a_w 0.999, ● = a_w 0.980 and ▲ = a_w 0.960.

Table 5.1: **Linear regression equations and a_{wmin} for *E. coli* SB1 in Nutrient Broth.**

Data Set	Linear regression equation	a_{wmin}
■ a_w 0.999 inoculum	y = -0.519929 + 0.544763x	0.954
● a_w 0.980 inoculum	y = -0.521095 + 0.544763x	0.957
▲ a_w 0.960 inoculum	y = -0.520742 + 0.544763x	0.955
All data below a_w 0.990	y = -0.520734 + 0.545717x	0.954

E. coli SB1-minimal laboratory media

For *E. coli* SB1 in 0.25NB-MM, a_w shifts were in the range of -0.046 to +0.036 a_w units. Raw experimental data are presented in A. 9.4.10. Growth rate versus a_w plots for the three inocula from different a_w are presented in Fig. 5.2 and exhibit similar, but not identical, growth rate responses to a_w.

Linear regression of the three plots in isolation and the data set as a whole for data below a_w 0.975, was undertaken using SAS PROC.GLM. Data below a_w 0.975 were selected due to the presence of aberrant points

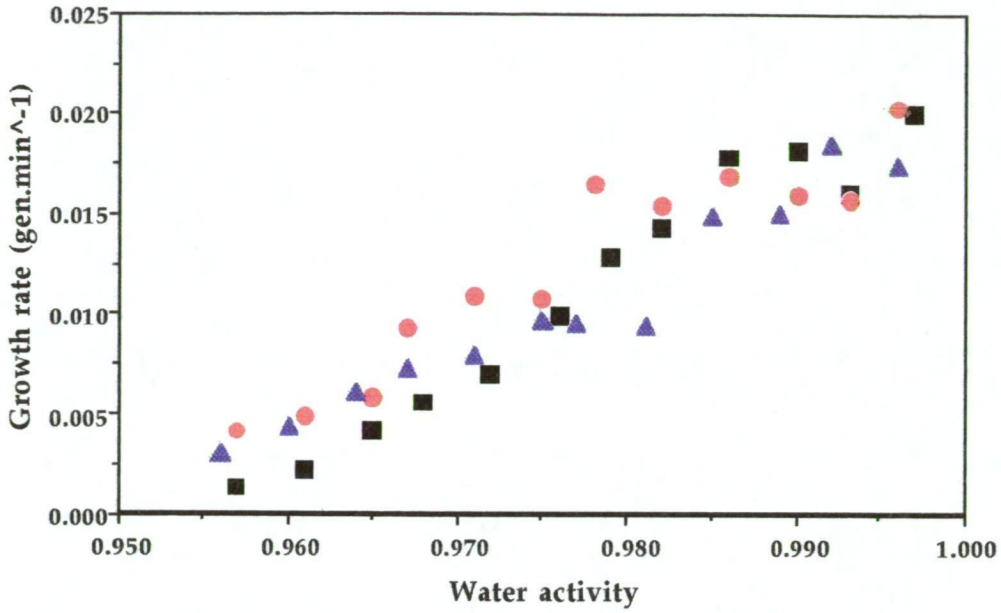

Figure 5.2: Effect of a_w shifts on the growth rate of *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium determined by turbidimetry, where: ■ = a_w 0.997, (●)= a_w 0.980 and ▲ = a_w 0.960.

for a_w 0.960 inocula above this a_w. The slopes of the regression lines were found not to be identical, however they were not significantly different (P=0.5210). The slope of each line was fixed to the common slope value to determine if the *x* axis intercepts were different. This analysis revealed that the intercepts were significantly different (P=0.0001). a_{wmin} values were calculated and are given in Table 5.2.

Table 5.2: Linear regression equations and a_{wmin} for *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium.

Data Set	Linear regression equation	a_{wmin}
■ a_w 0.997 inoculum	y = -0.369191 + 0.386928x	0.954
● a_w 0.980 inoculum	y = -0.366066 + 0.386928x	0.946
▲ a_w 0.960 inoculum	y = -0.367079 + 0.386928x	0.949
All data below a_w 0.975	y = -0.386872 + 0.407161x	0.950

E. coli R31-complex laboratory media

For *E. coli* R31 in NB, a_w shifts were in the range of -0.049 to +0.037 a_w units. Raw experimental data are presented in A. 9.4.11. Growth rate versus a_w plots for the three inocula from different a_w are presented in Fig. 5.3 and exhibit similar, but not identical, growth rate responses to a_w. For *E. coli* R31, curvature near the optimum a_w for growth was more pronounced for the a_w 0.980 inocula. Growth rate plots for a_w 0.999 and a_w 0.960 showed little curvature.

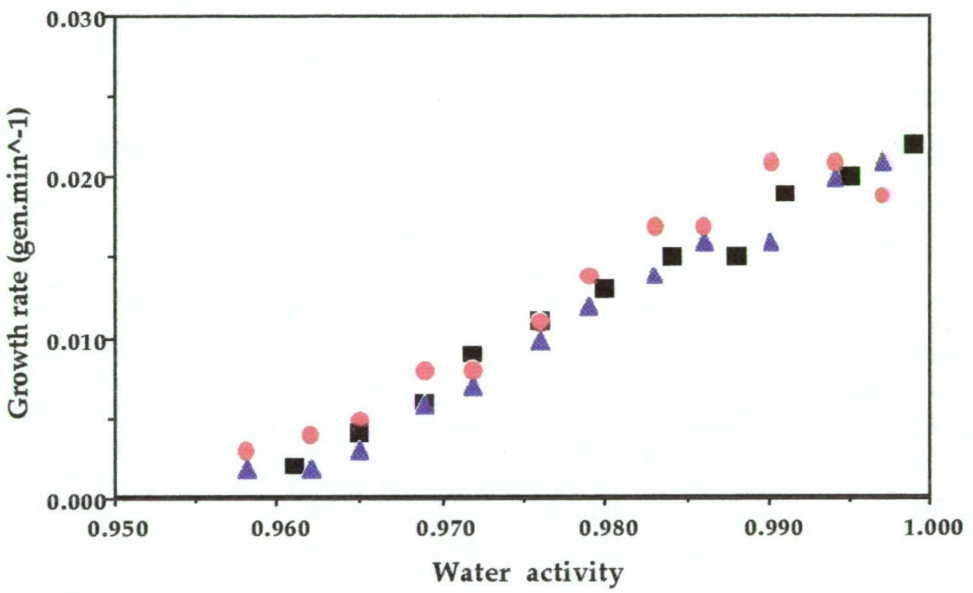

Figure 5.3: Effect of a_w shifts on the growth rate of *E. coli* R31 in Nutrient Broth determined by turbidimetry, where: ■ = a_w 0.997, (●)= a_w 0.980 and ▲ = a_w 0.960.

Linear regression of the three plots in isolation and the data set as a whole for data below a_w 0.990, was undertaken using SAS PROC.GLM. The slope of the regression lines were found not to be identical, however they were not significantly different (P=0.3453). The slope of each line was fixed to the common slope value to determine if the *x* axis intercepts were different. This analysis revealed that the intercepts were significantly different (P=0.0054). a_{wmin} values were calculated (Table 5.3).

Table 5.3: Linear regression equations and a_{wmin} for *E. coli* R31 in Nutrient Broth.

Data Set	Linear regression equation	a_{wmin}
■ a_w 0.997 inoculum	y = -0.480999 + 0.503270x	0.956
● a_w 0.980 inoculum	y = -0.479523 + 0.503270x	0.953
▲ a_w 0.960 inoculum	y = -0.481290 + 0.503270x	0.956
All data below a_w 0.990	y = -0.471189 + 0.493642x	0.955

K. oxytoca-complex laboratory media

For *K. oxytoca* in BHI, a_w shifts were in the range -0.054 to +0.046 a_w units. Raw experimental data are presented in A. 9.4.12. Growth rate versus a_w plots for the two inocula from different a_w are presented in Fig. 5.4 and exhibit similar, but not identical, growth rate responses to a_w. The response appears to be biphasic, with a change in slope occurring at ~ a_w 0.970.

Linear regression of the two plots in isolation and the data set as a whole for data below a_w 0.970, was undertaken using SAS PROC.GLM. The slopes of the regression lines were found not to be identical, however they were not significantly different (P=0.1331). The slope of each line was fixed to the common slope value to determine if the *x* axis intercepts were different. This analysis revealed that the intercepts were not significantly different (P=0.3941). a_{wmin} values were calculated and are given in Table 5.4.

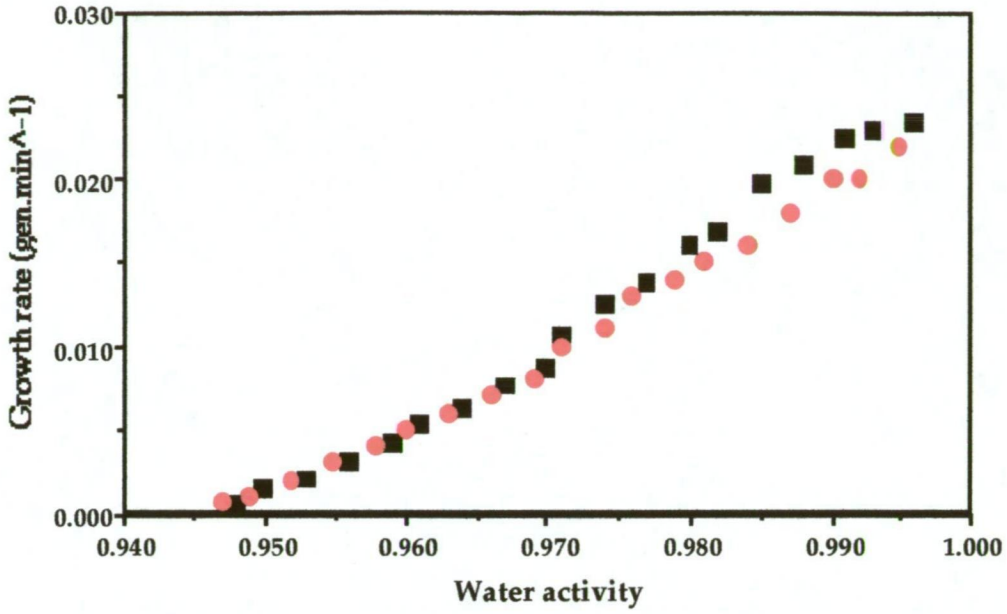

Figure 5.4: Effect of a_w shifts on the growth rate of *K. oxytoca* in Brain Heart Infusion Broth determined by turbidimetry, where: ■ = a_w 0.996 inoculum and ● = a_w 0.949 inoculum.

Table 5.4: Linear regression equations and a_{wmin} for *K. oxytoca* in Brain Heart Infusion Broth.

Data Set	Linear regression equation	a_{wmin}
■ a_w 0.999 inoculum	y = -0.335438 + 0.354426x	0.946
● a_w 0.949 inoculum	y = -0.335345 + 0.354426x	0.946
All data below a_w 0.970	y = -0.335591 + 0.354638x	0.946

Effect of immediate NaCl mediated a_w shifts on RLT of *E. coli* SB1, *E. coli* R31, *K. oxytoca*, *S.* Typhimurium, *S. aureus* and *L. monocytogenes*

Relative lag time is the ratio of lag time divided by the generation time. It provides a measure of the *amount* of work to be done by a cell before growth is initiated in a new environment. To provide data on *near minimum* physiological lag times, i.e. a lag time induced mainly in response to an abrupt change in environment as opposed to resolution of a resting stage, osmotic shifts were applied instantaneously to late exponential phase cultures of *E. coli* SB1, *E. coli* R31, *K. oxytoca*, *S.* Typhimurium, *L. monocytogenes* and *S. aureus*. The type of a_w shift applied to the cultures were those detailed in S. 5.2.1.1.

In general, the RLT was extended by increasingly large shifts in a_w. The effect was more pronounced for cultures subjected to a a_w downshift. Gram negative organisms subjected to a a_w downshift displayed a more complex RLT response than the gram positive organisms.

E. coli SB1-complex laboratory media

The RLT response for *E. coli* SB1 in NB was dependent on the osmotic history of the exponentially growing inoculum (Figure 5.5). Raw experimental data are presented in A. 9.4.9.

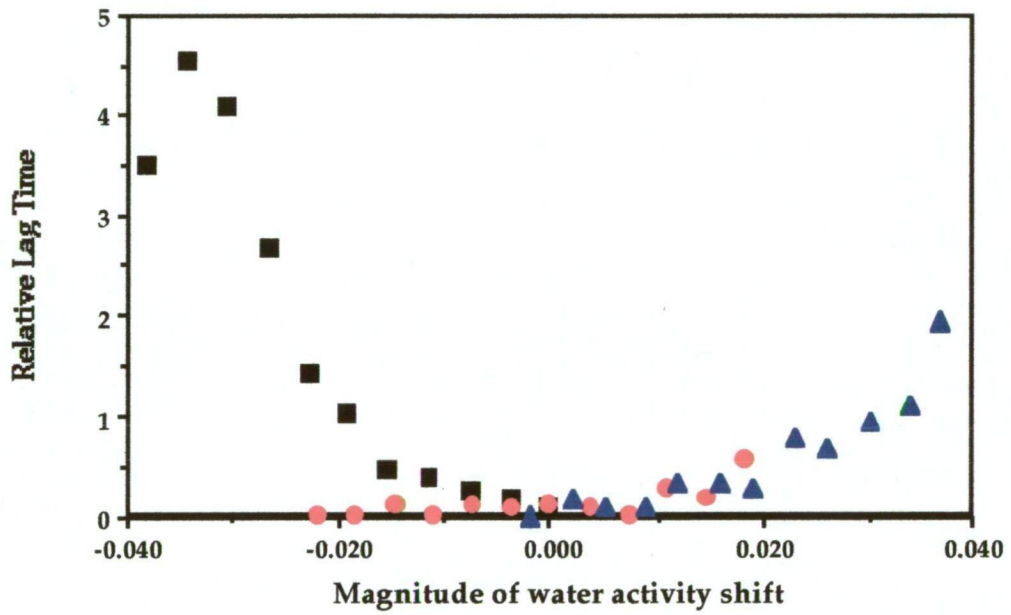

Figure 5.5: Effect of a_w shifts on the relative lag time response of *E. coli* SB1 in Nutrient Broth determined by turbidimetry, where: ■ = a_w 0.999, ● = a_w 0.980 and ▲ = a_w 0.960.

An upshift from a_w 0.960 resulted in RLTs increasing approximately in direct proportion to increasing a_w. For shifts from the middle of the growth permissive range for a_w (0.980), i.e. a_w upshifts and downshifts, RLT showed little change and was <0.6 in all cases. A downshift from a_w 0.999 produced a more complex RLT response, with RLT increasing approximately in direct proportion to decreasing a_w for shifts of -0.015 a_w units or less. For larger shifts the RLT response then increased up to an

RLT of 4.5, which corresponded to a shift of -0.034 a_w units. At downshifts >0.034 a_w units RLT then decreased.

E. coli SB1-minimal laboratory media

The RLT response for *E. coli* SB1 in minimal media was similar to that in complex media, i.e. it was dependent on the osmotic history of the exponentially growing inoculum (Figure 5.6). Raw experimental data are presented in A. 9.4.10.

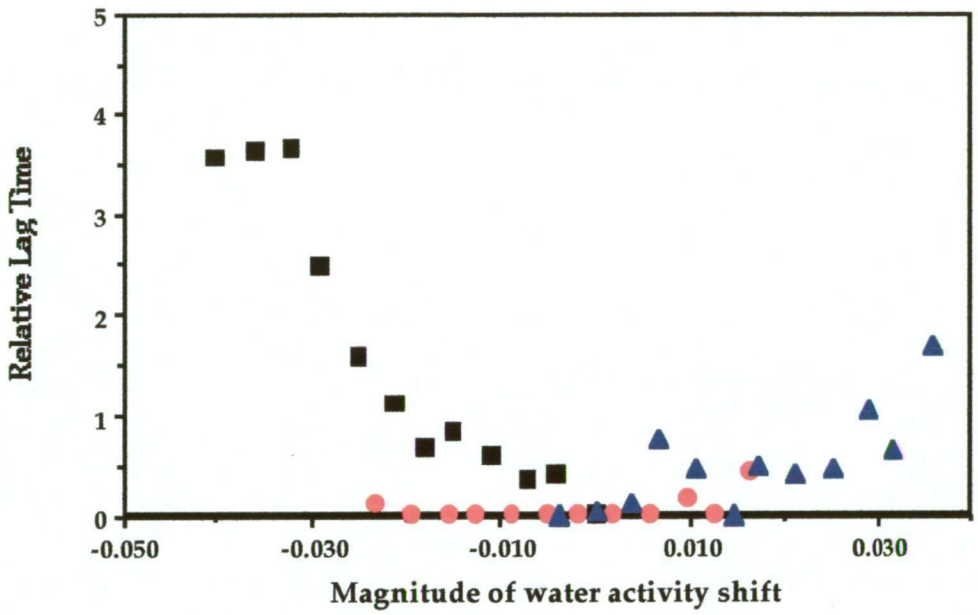

Figure 5.6: Effect of a_w shifts on the relative lag time response of *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium determined by turbidimetry, where: ■ = a_w 0.999, ● = a_w 0.980 and ▲ = a_w 0.960.

An upshift from a_w 0.960 resulted in RLTs increasing approximately in direct proportion to increasing a_w. For a shift from the middle of the growth permissive range for a_w (0.980), RLT showed little response and were all <0.5. A downshift from a_w 0.997 produced a more complex RLT response, with RLT increasing approximately in direction proportion to decreasing a_w until a shift of -0.032 a_w units, which corresponded to a RLT of 4.1. More extreme downshifts resulted in smaller RLTs.

E. coli R31-complex laboratory media

The RLT response for *E. coli* R31 was similar to that for *E. coli* SB1, however RLTs were generally smaller (Figure 5.7). Raw experimental data are presented in A. 9.4.11.

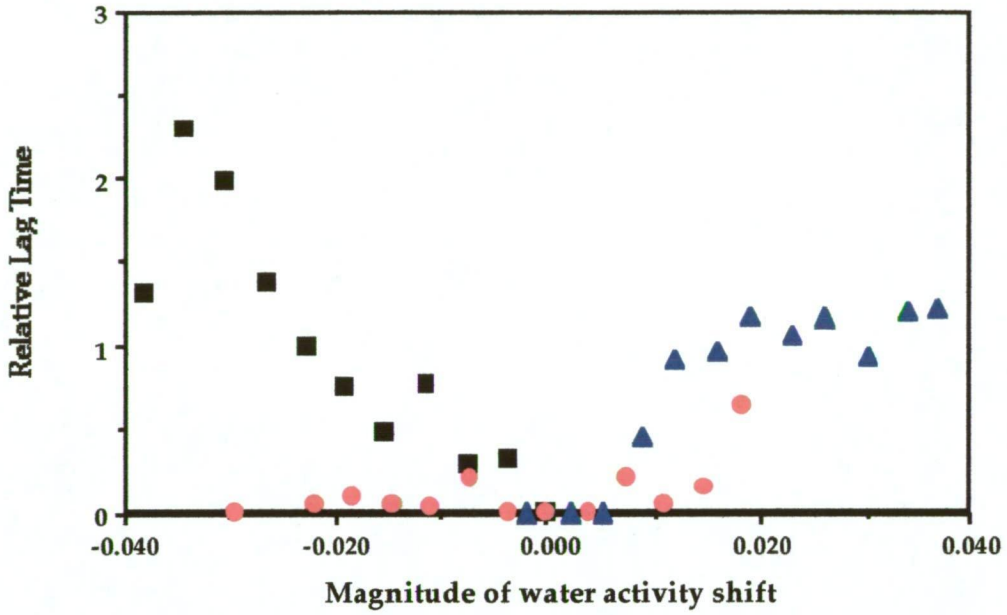

Figure 5.7: Effect of a_w shifts on the relative lag time response of *E. coli* R31 in Nutrient Broth determined by turbidimetry, where: ■ = a_w 0.999, ● = a_w 0.980 and ▲ = a_w 0.960.

An upshift from a_w 0.960 resulted in RLTs increasing approximately in proportion to increasing a_w. For shifts from the middle of the growth permissive range for a_w (0.980), i.e. a_w upshifts and downshifts, RLT showed little change and was <0.7 in all cases. A downshift from a_w 0.999 produced a more complex RLT response, with RLT increasing approximately in direct proportion to decreasing a_w for shifts of -0.034 a_w units or less, up to a RLT of 2.3. At downshifts >0.034 a_w units RLT then decreased.

K. oxytoca-complex laboratory media

For turbidimetric determinations of RLT for *K. oxytoca*, the response for a a_w upshift was different to that for a a_w downshift (Figure 5.8). Raw experimental data are presented in A. 9.4.12.

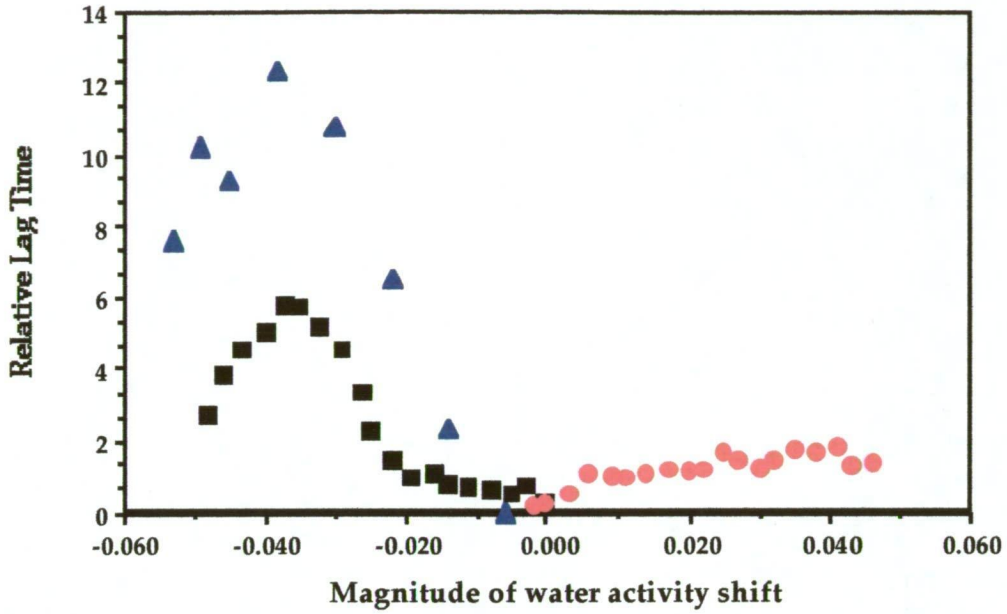

Figure 5.8: Effect of a_w shifts on the relative lag time response of *K. oxytoca* in Brain Heart Infusion Broth determined by turbidimetry: ■ = a_w 0.996, ● = a_w 0.949, and by viable count (▲) where inoculum a_w = 0.998.

For an upshift, RLT increased approximately in direct proportion to increasing a_w, with all RLTs remaining <2. For a downshift, the RLT response was more complex. RLT increased approximately in direction proportion to decreasing a_w until a shift of -0.022 a_w units. For larger shifts the RLT response then increased up to a RLT of 5.7, which corresponded to a shift of -0.037 a_w units. At greater downshifts RLT decreased. It should be noted that the RLT datum point for a -0.003 a_w unit shift (corresponding to a_w 0.993) appears aberrant if the a_w downshift data are plotted in isolation. The RLT value is higher than for the data surrounding it. The generation time estimate from this condition does not appear unusual (*see* Fig 5.4), suggesting that experimental error may have occurred in the lag time estimation only.

Viable count assays showed a response similar to that for optical density for a_w downshifts, with RLT increasing up to 12.4, corresponding to a shift of -0.038 a_w units, after which RLT decreased to 7.6. Raw experimental data are presented in A. 9.4.13.

K. oxytoca-minimal media

For *K. oxytoca* in 0.20NB-MM, the RLT response was similar to that in the complex laboratory media (Figure 5.9). Raw experimental data are presented in A. 9.4.14. RLT increased approximately in direct proportion to decreasing a_w until a shift of -0.038 a_w units (RLT of 2.5). For larger shifts the RLT response increased up to a RLT of 2.7. At greater downshifts the RLT decreased.

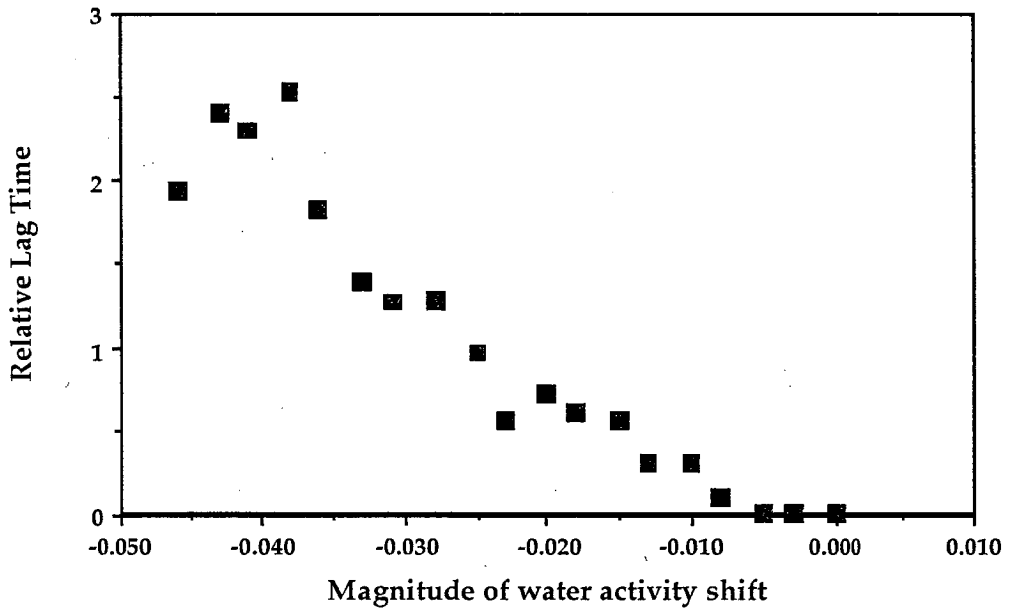

Figure 5.9: Effect of a_w shifts on the relative lag time response of *K. oxytoca* (■) in 1/5 strength Nutrient Minimal Medium, determined by turbidimetry.

S. **Typhimurium** *–complex laboratory media*

The RLT response for *S.* Typhimurium to a a_w downshift is similar to that for *K. oxytoca* in complex laboratory media (Fig. 5.10). Raw experimental data are presented in A. 9.4.15. RLT increased approximately in direct proportion to decreasing a_w until a shift of -0.020 a_w units. For larger shifts the RLT response then increased up to a RLT of ~6.2, which corresponded to a shift of -0.027 a_w units. At greater downshifts RLT decreased.

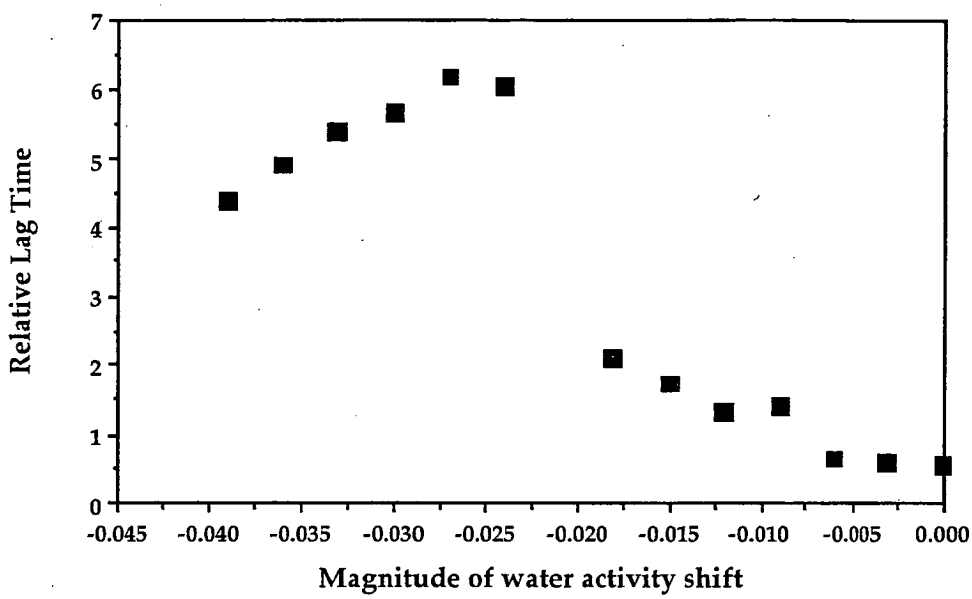

Figure 5.10: Effect of a_w downshift on the relative lag time response of *S.* Typhimurium in Brain Heart Infusion Broth, a_w 0.993 (●), determined by turbidimetry.

S. aureus–complex laboratory media

After a a_w downshift the RLTs of *S. aureus* were generally unaffected over the range of a_w tested, remaining <0.35 (Fig. 5.11). Raw experimental data are presented in A 9.4.16.

L. monocytogenes–complex laboratory media

After a a_w downshift the RLTs of *L. monocytogenes* were generally unaffected over most of the range of a_w tested, approximately 0.5 (Figure 5.11). Close to the a_w limits for growth, RLT increased up to 1.6. Raw experimental data are presented in A. 9.4.17.

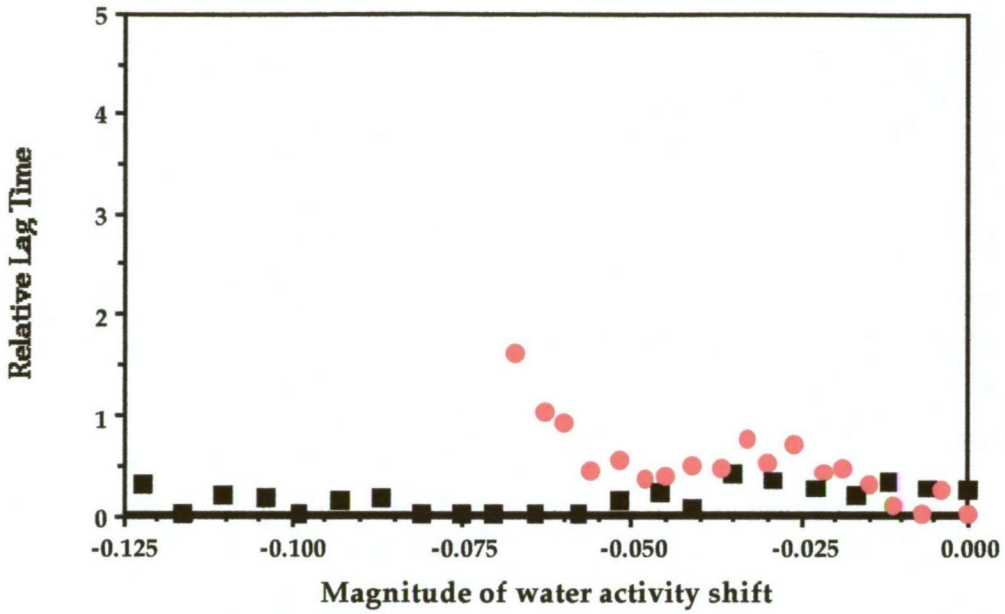

Figure 5.11: Effect of a_w downshifts on the relative lag time responses of *S. aureus* in Nutrient Broth, a_w 0.998, (■) and *L. monocytogenes* in Tryptone Soya Broth with 0.6% Yeast Extract added, a_w 0.996, (●), determined by turbidimetry.

Effect of immediate osmotic shifts on bacterial lag rate

Lag rate, the reciprocal of lag time in minutes, was calculated from the results of a_w upshift and downshift experiments involving gram negative organisms. Lag rate was calculated to examine that part of the relative lag time concept that involves the *amount* of work to be done to resolve the lag phase.

E. coli SB1

For a a_w downshift, the lag rate for *E. coli* SB1 decreased with decreasing a_w in both complex (Fig. 5.12) and minimal (Fig. 5.13) laboratory media. The plots for the a_w downshift are notably not biphasic. The response was more curvilinear. For an upshift, the lag rate response in the complex and minimal medium showed no easily discernible trend. In complex media, although lag rate appeared to decrease slowly with increasing a_w, linear regression analysis using Cricket Graph v.1.3.2 showed this response was not statistically significant ($y = 4.7262e\text{-}2 - 0.91283x$, $R^2 = 0.365$). This was

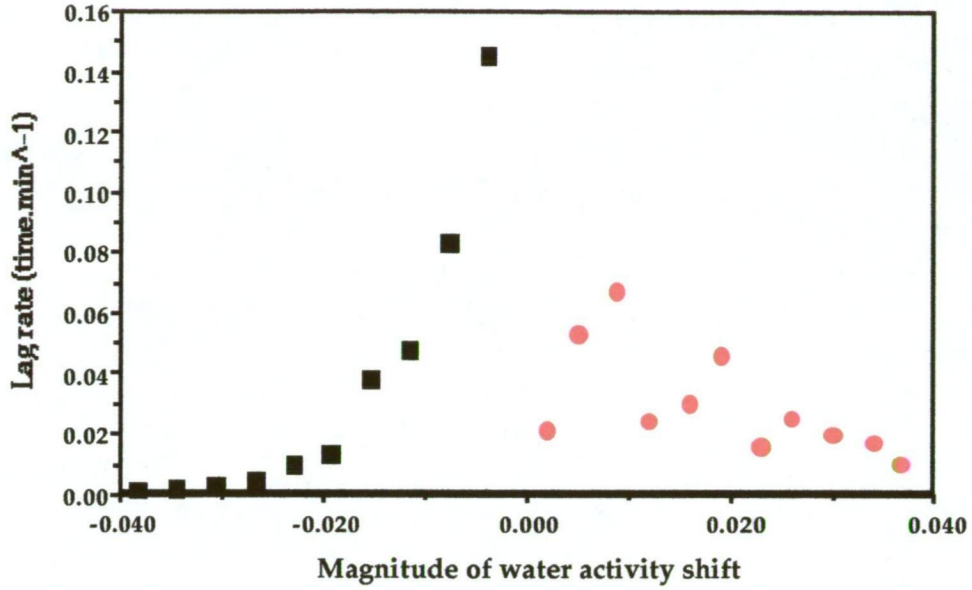

Figure 5.12: Effect of a_w shifts on the lag rate of *E. coli* SB1 in Nutrient Broth, determined by turbidimetry where (■) = downshift and (●) = upshift.

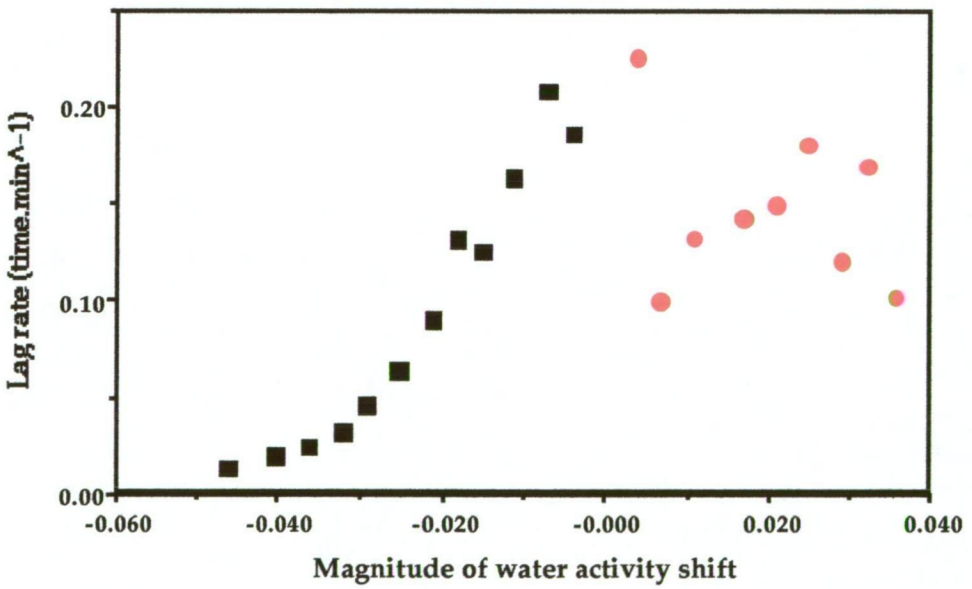

Figure 5.13: Effect of a_w shifts on the lag rate of *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium determined by turbidimetry, where: (■)= downshift and (●) =upshift

also the case for the lag rate response for a a_w upshift in minimal media (y = 0.16608 - 0.97585x, R^2 = 0.075).

E. coli R31

The lag rate response to a a_w downshift for *E. coli* R31 is similar to that for *E. coli* SB1, particularly at lower a_w (Fig. 5.14). For an upshift, lag rate increased slightly with increasing a_w.

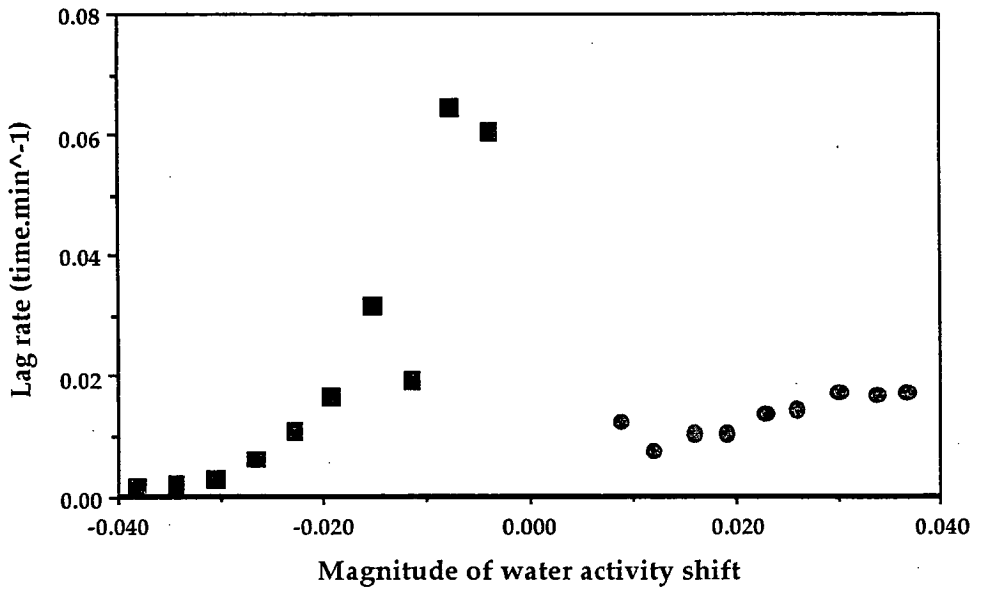

Figure 5.14: Effect of a_w shifts on the lag rate of *E. coli* R31 in Nutrient Broth, as determined by turbidimetry where (■) = downshift and (●) = upshift.

K. oxytoca

For a a_w downshift in a complex laboratory medium, the lag rate for *K. oxytoca* decreased with decreasing a_w (Fig. 5.15). For the turbidimetric determinations in the complex medium the response appeared to be biphasic, with a change in the rate of decrease of lag rate occurring at a shift of -0.0026 a_w units (corresponding to a_w 0.970). It should be noted that a lag rate datum point for a 0.000 a_w shift, i.e. the culture was shifted to fresh broth of the same a_w, was included in Fig. 5.15. This was to highlight the linear decrease in lag rate as the datum point for a -0.003 a_w unit shift (corresponding to a_w 0.993) appears aberrant. The irregularity of this datum point was also noted in S. 5.3.1.1 (*see* Fig. 5.4).

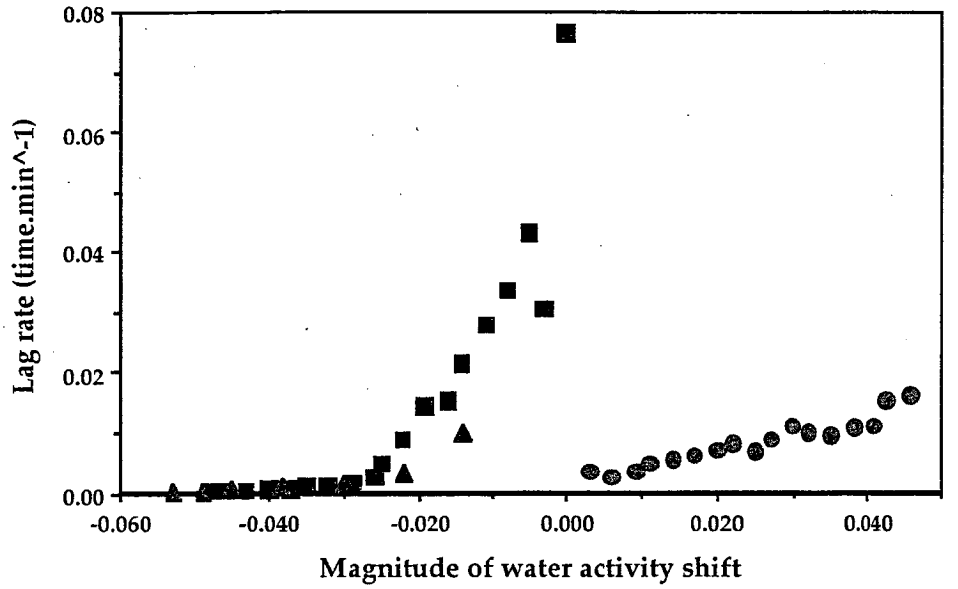

Figure 5.15: Effect of a_w shifts on the lag rate of *K. oxytoca* in Brain Heart Infusion Broth determined by turbidimetry where (■) = downshift, (●) = upshift and (▲) = downshift (results by viable count).

The lag rate response for a a_w downshift determined by viable count appears similar to that observed for turbidimetric determinations, i.e. a biphasic pattern. For a a_w upshift in a complex medium, lag rate increased linearly with increasing a_w (y=1.5112e-3 + 0.27387x, R^2 0.900). The scale over which lag rate increased was narrower for a a_w upshift than for a downshift in the complex medium.

For a a_w downshift in a 0.20NB-MM, the lag rate response for *K. oxytoca* was similar to that observed in a complex medium (Fig. 5.16).

S. Typhimurium

For a a_w downshift in a complex laboratory medium, the lag rate for S. Typhimurium was similar to that for *K. oxytoca*. Lag rate decreased with decreasing a_w (Fig. 5.17). The response appeared to be biphasic, with a change in the rate of decrease of lag rate occurring at a shift of ~ -0.0020 a_w units (corresponding to a_w ~0.970).

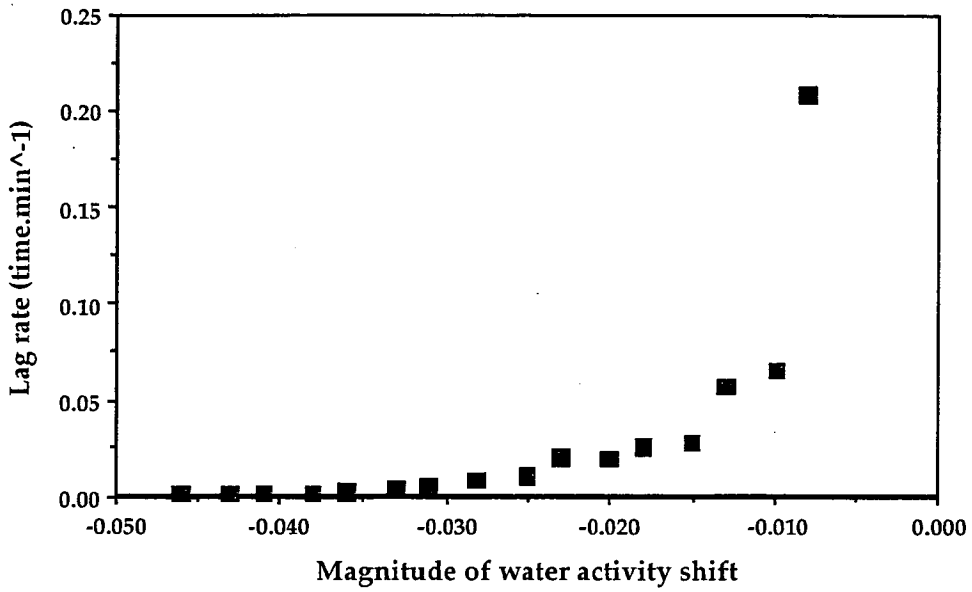

Figure 5.16: Effect of a_w downshift on the lag rate of *K. oxytoca* in 1/5 strength Nutrient Minimal Medium (■), determined by turbidimetry.

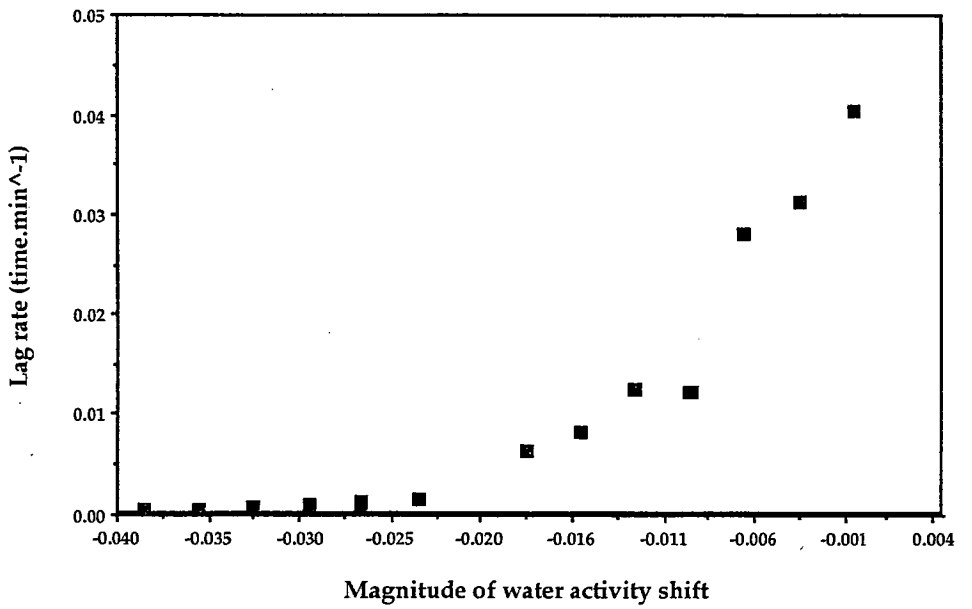

Figure 5.17: Effect of a_w downshift on the lag rate of *S.* Typhimurium in Brain Heart Infusion broth (■), determined by turbidimetry.

Effect of immediate osmotic shifts on 'cell yield' of gram-negative bacteria

To investigate whether the a_w of the inoculum affected the 'cell yield' of *E. coli* SB1 and *K. oxytoca*, exponentially growing cells were subjected to immediate osmotic shifts in minimal media. The type of a_w shift applied to the cultures are detailed in S. 5.2.1. Growth of the cells was monitored by measurement of OD in the new osmotic environment and 'cell yield' was calculated from the difference between the final and initial optical density.

E. coli SB1

The osmotic history of the inoculum did not appear to affect the 'cell yield' response for *E. coli* SB1 over the growth permissive range for a_w (Fig. 5.18). 'Cell yield' data are presented in A. 9.4.10.

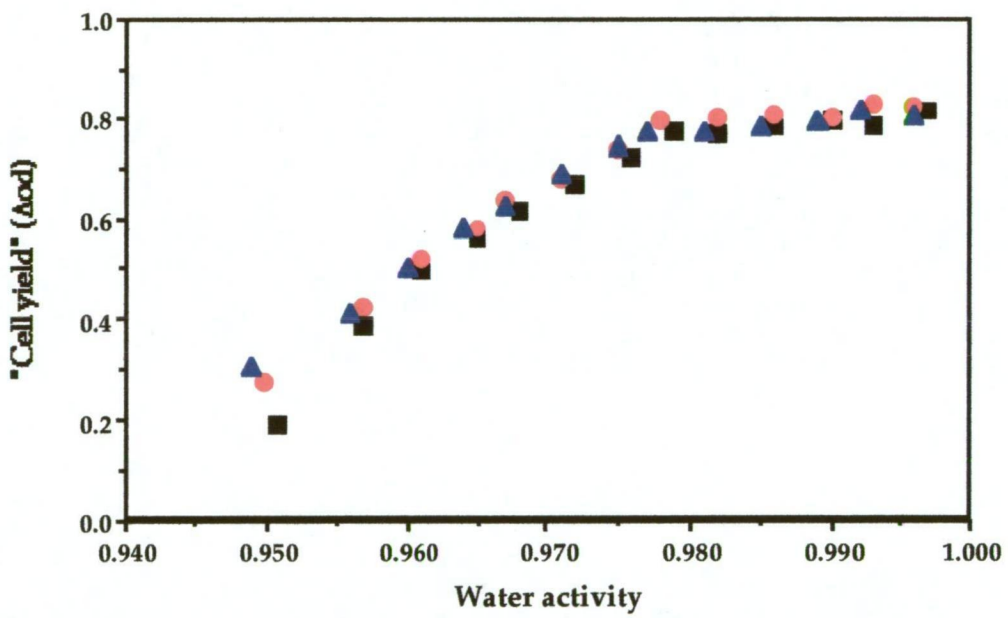

Figure 5.18: Effect of a_w shifts on the 'cell yield' of *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium determined by turbidimetry, where: ■ = a_w 0.997, ● = a_w 0.980 and ▲ = a_w 0.960.

Some differences occurred near the lower a_w limit for growth, but it should be noted that growth was non-sustainable at these a_ws and growth rate could not be estimated. 'Cell yield' remained unaffected until a_w0.975; 'cell yield' then began to decline for all three pre-inoculation treatments.

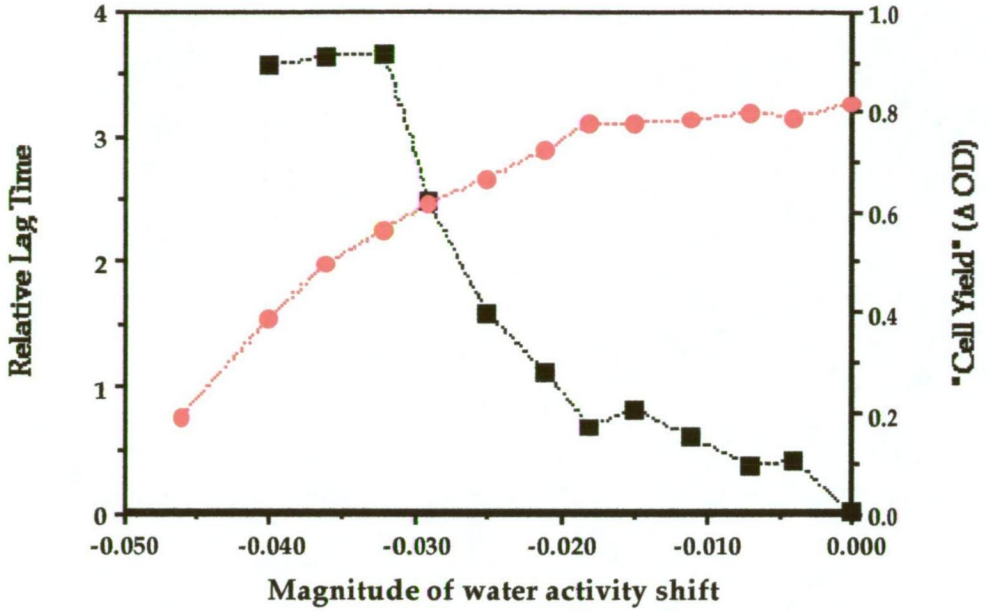

Figure 5.19: Effect of a_w downshift on the relative lag time (■) and 'cell yield' (●) of *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium determined by turbidimetry.

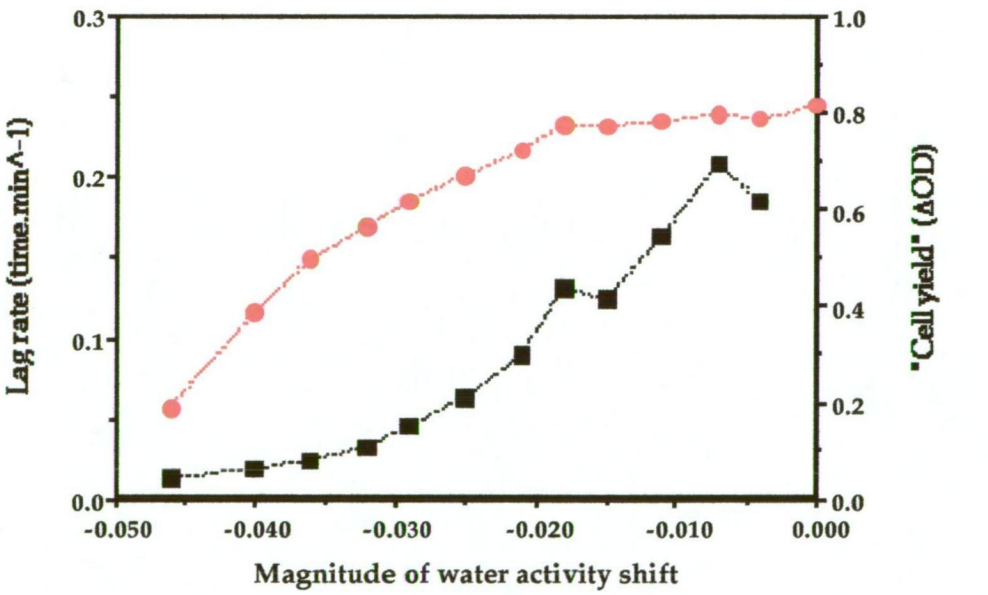

Figure 5.20: Effect of a_w downshift on the lag rate (■) and 'cell yield' (●) of *E. coli* SB1 in 1/4 strength Nutrient Minimal Medium determined by turbidimetry.

For a a_w downshift, the a_w at which yield begins to decline, ~a_w 0.975, coincides with the rapid increase observed in RLT after a downshift of -.0.015 a_w units (Fig. 5.19). No clear relationship was observed when 'cell yield' was plotted with lag rate (Fig. 5.20).

K. oxytoca

'Cell yield' of *K. oxytoca* was measured in response to a a_w downshift only, with 'cell yield' beginning to decline at about a_w 0.980 (Fig. 5.21). 'Cell yield' data are presented in A.9.4.14.

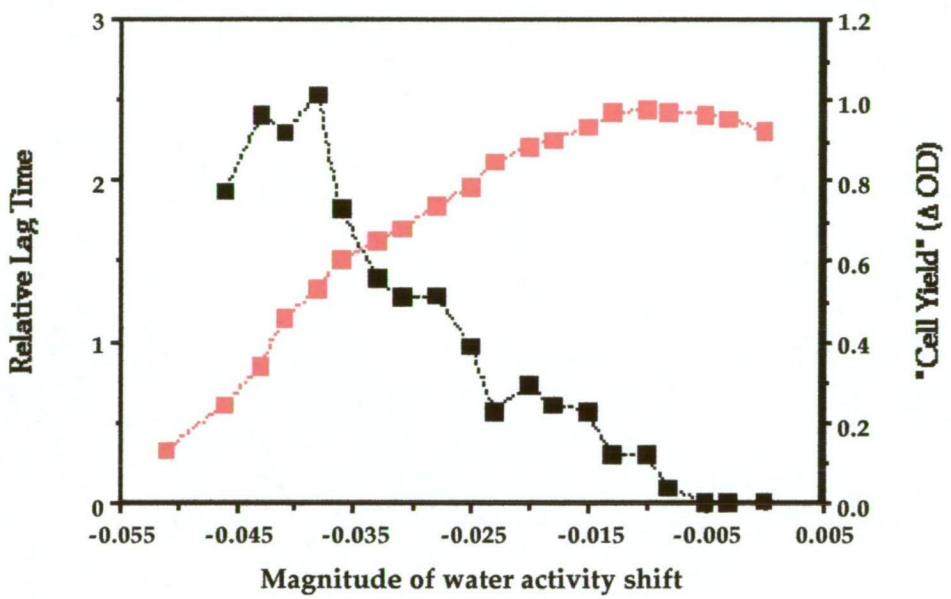

Figure 5.21: Effect of a_w downshift on the relative lag time (■) and 'cell yield' (●) of *K. oxytoca* in 1/5 strength Nutrient Minimal Medium determined by turbidimetry

There appears to be no clear relationship when plotted with RLT, however the response is not inconsistent with that observed for *E. coli* SB1. In general, RLT increases as the magnitude of the osmotic shift increases, and where 'cell yield' falls coincides with an increase in RLT.

When 'cell yield' was plotted with lag rate, the value where yield began to decline coincided with a change in the lag rate (Fig. 5.22).

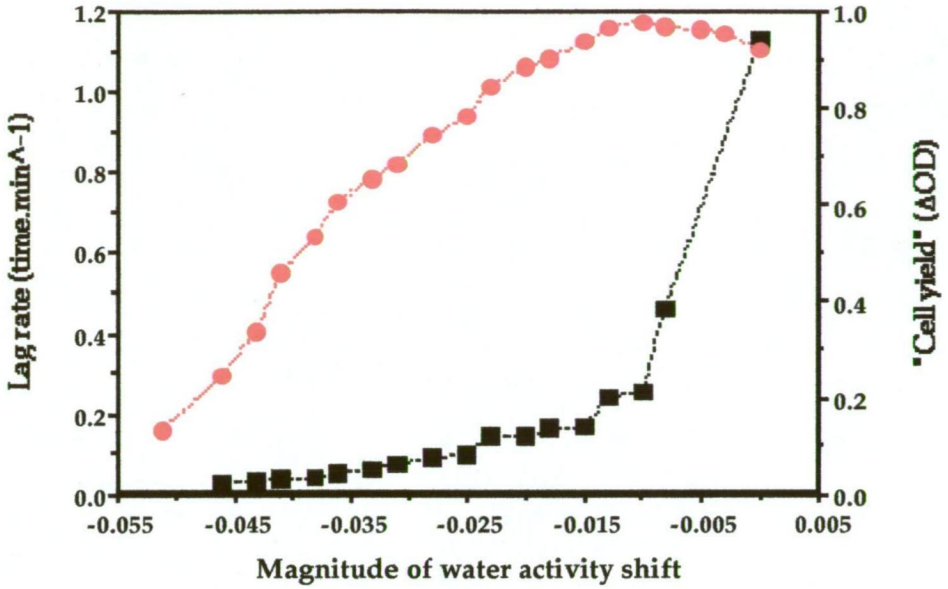

Figure 5.22: Effect of a_w downshift on the lag rate (■) and 'cell yield' (●) of *K. oxytoca* in 1/5 strength Nutrient Minimal Medium determined by turbidimetry

5.3.1.2 Potential a_w mediated sub-lethal injury in *K. oxytoca*

Preliminary results for growth on non-selective PCA and selective MAC

During experiments to confirm turbidimetric observations of RLT (*see* S. 5.2.1.3), cultures in three broths of a_w 0.992, 0.976 and 0.960 were plated onto the selective medium MAC.

After inoculation into broth at a_w 0.992, growth of *K. oxytoca* resumed immediately, as determined by viable count on PCA (Fig. 5.23). For counts determined on MAC, there was a 3 log decrease in colony forming units. Counts on MAC quickly returned, i.e. after 50 minutes, to levels similar to the starting numbers. For the remainder of the growth curve, counts on MAC were slightly lower than that for PCA until the stationary phase of growth was reached.

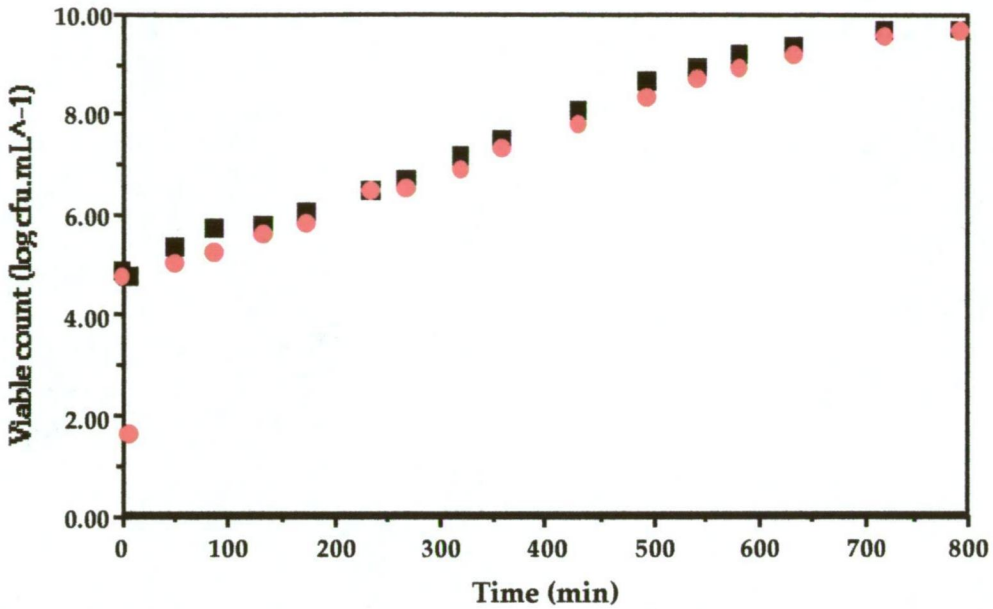

Figure 5.23: Growth of *K. oxytoca* in Brain Heart Infusion Broth at a_w 0.992 determined by viable count on Plate Count Agar (■) and MacConkey Agar (●).

Immediately after inoculation into broth at a_w 0.976, a 2 log decrease in colony forming units was observed on PCA, and a >4 log decrease on MAC (Fig. 5.24). The initial decrease observed on MAC was below the level of detection for the spread plate technique used, i.e.<10 cfu.mL^{-1}. These observations are indicators of the extent of the apparent injury to the cells, and have been plotted as open symbols. Counts on PCA quickly returned, i.e. after 100 minutes, to levels similar to the number at the time of inoculation. Counts on MAC returned to the starting number more slowly, i.e. after approximately 130 minutes. For the remainder of the growth curve, counts were similar on PCA and MAC.

Immediately after inoculation into broth at a_w 0.960, a 2 log decrease in colony forming units was observed on PCA, and a 4 log decrease on MAC (Fig. 5.25). Counts on PCA slowly returned, i.e. after 1000 minutes, to levels similar to the starting number after inoculation. Counts on MAC returned to the starting number after a similar time period. In the early stages of the growth curve, counts on MAC were

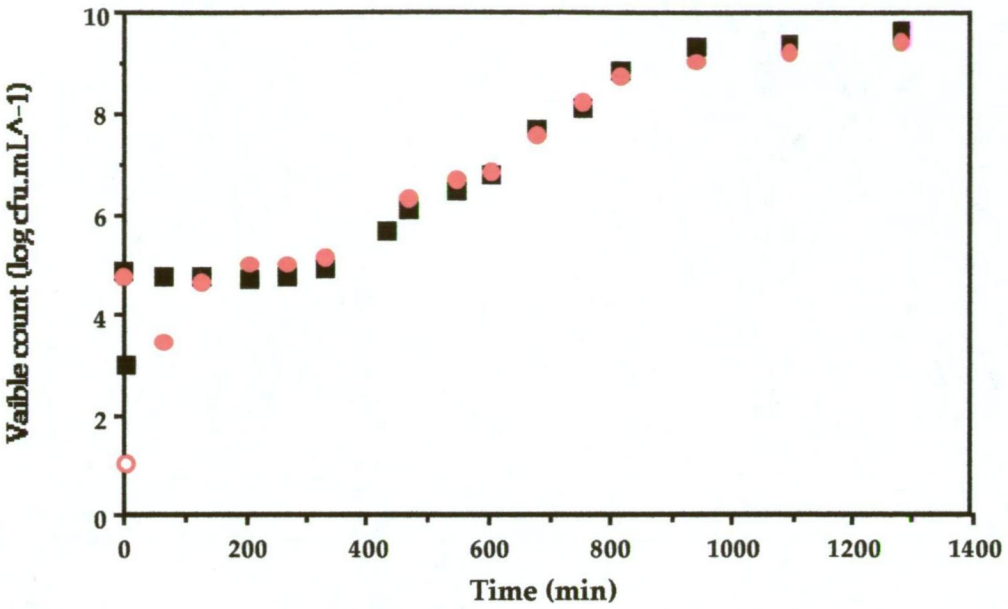

Figure 5.24: Growth of *K. oxytoca* in Brain Heart Infusion Broth at a_w 0.976 determined by viable count on Plate Count Agar (■), MacConkey Agar (●) and counts on MacConkey Agar that were <10 cfu.mL^{-1} (○).

lower than on PCA. Counts returned to levels similar to PCA after 750 minutes. For the remainder of the growth curve, counts were similar on PCA and MAC.

Assessment of recovery conditions for investigating a_w mediated sublethal injury

The results presented above, showing differences in colony forming units recovered on the non-selective PCA and selective MAC, suggest that shifting cells to lower a_w may cause injury. Growth curves were altered, which may have implications for calculation of generation and lag times. A more detailed investigation was undertaken to determine if the apparent injury to cells after a large and abrupt shift in osmotic environment was an artifact of media type, diluent and incubation temperature, or a genuine reflection of a temporary loss of culturability (i.e. injury). The effect on generation and lag time calculations was examined.

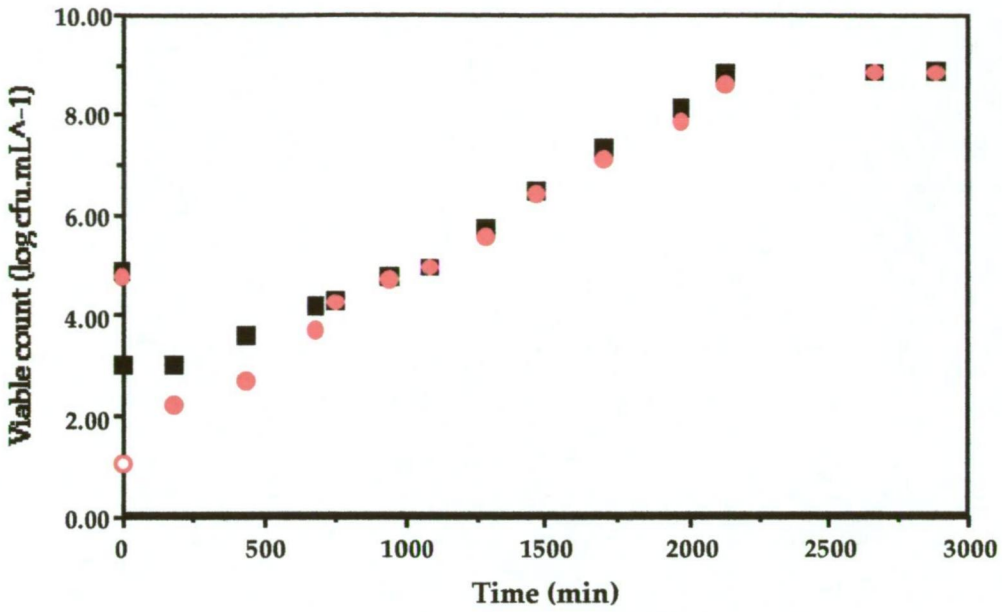

Figure 5.25: Growth of *K. oxytoca* in Brain Heart Infusion Broth at a_w 0.960 determined by viable count on Plate Count Agar (■), MacConkey Agar (●) and counts on MacConkey Agar that were <10 cfu.mL^{-1} (○).

Viable count data for growth of *K. oxytoca*, after a shift from a_w 0.997 to a_w 0.962 at 25°C, on a variety of media and using PW diluent are shown in Figure 5.26. Temperature of incubation is recorded in brackets. Some sampling times yielded no counts on MAC as they were below the level of detection of the spread plate technique, i.e. <10 cfu.mL^{-1}. They are indicated by open symbols.

Immediately after inoculation of the test broth, colony counts decreased on all media types. For cultures plated on PCA (25°C) and BHAP, the reduction was approximately 1 log. A 2 log reduction was observed for PCA (37°C) and a >4 log decrease was observed on MAC at both 25 and 37°C. Counts on the non-selective media were similar throughout the growth curve. Counts on MAC at both incubation temperatures were much lower than on the non-selective media in the early phases of growth. In general they remained lower than counts on the non-selective media through the exponential phase. As the culture neared

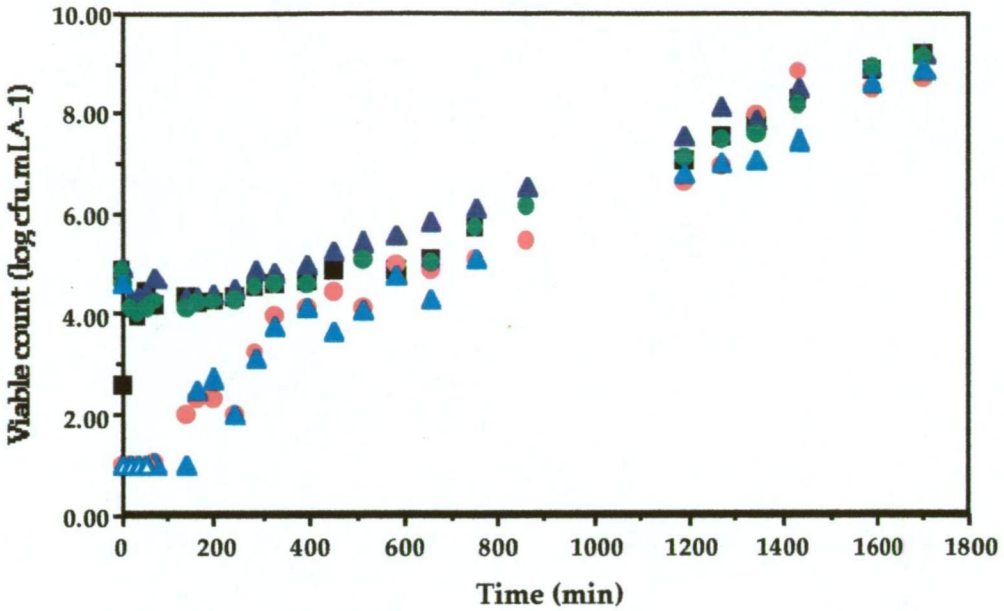

Figure 5.26: Growth of *K. oxytoca* at a_w 0.962 determined by viable count using 0.1% Peptone Water as diluent, where; ■ = Plate Count Agar (37°C), ● = Plate Count Agar (25°C), ▲ = Brain Heart Infusion Agar with 0.1% pyruvate added (25°C), ● = MacConkey Agar (37°C), ○ = counts on MacConkey Agar (37°C) <10 cfu.mL^{-1}, ▲ = MacConkey Agar (25°C) and Δ = counts on MacConkey Agar (25°C) <10 cfu.mL^{-1}.

stationary phase the counts on all media type and incubation conditions became similar.

Analysis of the growth curves by linear regression, S. 2.1.3.3, provided information on generation and lag times (Table 5.5). Generation time estimates for the experiment with PW as diluent varied little between the agar/incubation conditions. Lag time estimates were affected. The shortest lag time estimate was determined on BHAP and the longest on MAC at both incubation temperatures. Lag time estimates were similar for PCA at both incubation temperatures. This was true also for MAC. Differences in the lag time estimates also affected RLT calculations.

Table 5.5: Generation and lag time estimates for growth of *K. oxytoca* at a_w 0.962, 25°C on various media with 0.1% Peptone Water as diluent. Relative lag times were also calculated.

Medium	GT (min)	Lag time (min)	RLT
■ = PCA(37°C)	77.1	584.9	7.6
● = PCA(25°C)	83.2	534.4	6.4
▲ = BHAP(25°C)	91.5	355.9	3.9
● = MAC (37°C)	81.8	647.9	7.9
▲ = MAC (25°C)	79.5	675.4	8.5
Average	**82.6**	**559.7**	**6.8**
Standard deviation	**± 4.3**	**± 126.5**	**± 1.8**

The possibility that the diluent or the recovery medium induces an additional osmotic shock was investigated by using a diluent of similar a_w to the culture medium and incorporating NaCl into a non-selective medium, i.e. to avoid osmotic shocks on transfer from the diluent. Viable count data for growth of *K. oxytoca* after a shift from a_w 0.997 to a_w 0.962 at 25°C on a variety of media, all incubated at 25°C, with PWS diluent are shown in Figure 5.27. Some sampling times yielded no counts on MAC as they were below the level of detection of the spread plate technique. They are indicated by open symbols.

After inoculation, counts decreased on all media types. For PCA+5% NaCl, PCA and BHAP a 1 log decrease occurred. A >4 log reduction was observed on MAC. Counts on the non-selective media were similar throughout the growth curve. Counts on MAC were much lower than the non-selective media in the early phases of growth. They remained lower than counts on the non-selective media for the remainder of the growth curve.

Analysis of the growth curves by linear regression, S. 2.1.3.3, provided information on generation and lag times (Table 5.6). Generation time estimates for the experiment with PWS diluent varied little between the agar types. Lag time estimates were similar for PCA, PCA+5% NaCl and BHAP. MAC had a longer lag time estimate. The variation in lag time estimates, and thus RLT calculations, was less than for the corresponding experiment with PW as diluent.

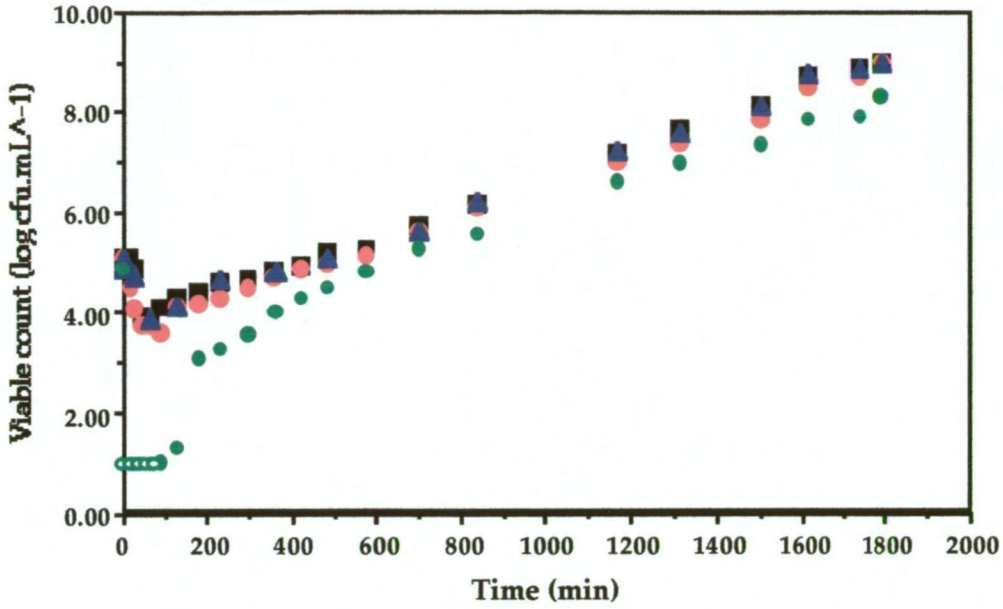

Figure 5.27: Growth of *K. oxytoca* at a_w 0.962 determined by viable count using 0.1% Peptone Water with 5%NaCl added as diluent on media incubated at 25°C for 48 hours where: ■ = Plate Count Agar with 5% NaCl added, ● = Plate Count Agar, ▲ = Brain Heart Infusion Agar with 0.1% pyruvate added, ● = MacConkey Agar and ○ = counts on MacConkey Agar <10 cfu.mL-1.

Table 5.6: Generation time and lag time estimates for growth of *K. oxytoca* at a_w 0.962, 25°C on various media with 0.1% Peptone Water with 5%NaCl added as diluent. Relative lag times were calculated.

Medium	GT (min)	Lag time (min)	RLT
■ = PCA+5% NaCl	98.4	408.3	4.2
● = PCA	100.4	446.1	4.4
▲ = BHAP	99.7	396.2	3.4
● = MAC	110.9	550.1	5.0
Average	102.4	450.2	4.4
Standard deviation	± 5.0	± 60.6	± 0.5

5.3.2 Temperature Shift

5.3.2.1 Effect of temperature shifts on *E. coli* SB1

Effect on the growth rate of *E. coli* SB1

Cultures were subjected to abrupt temperature shifts in the range of ΔT=-34.5 to +45.5 °C (*see* S. 5.2.2.1). Raw experimental data are presented in A. 9.4.18. Growth curves for the two exponentially prepared inocula at different temperature are presented in Figure 5.28.

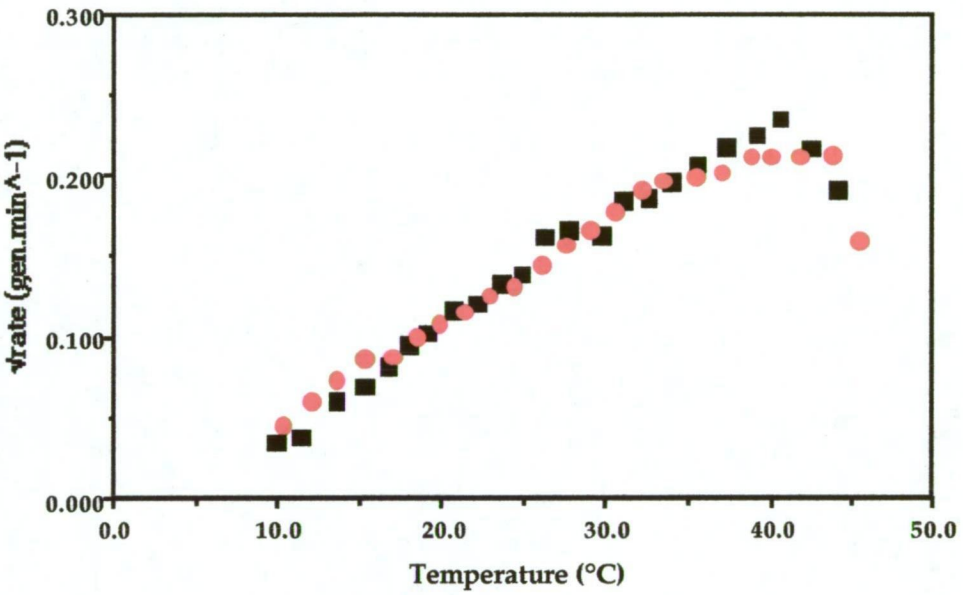

Figure 5.28: Effect of temperature shifts on the square root of growth rate of *E. coli* SB1 in Nutrient Broth determined by turbidimetry, where: ■ = 44.4 (± 0.1°C) inoculum and ● = 10.0 (± 0.1°C) inoculum.

Curvature near the optimum temperature for growth was sharper for the 44.4 (± 0.1°C) inoculum and less pronounced for the 10.0 (± 0.1°C) inoculum. Eqn (2), was fitted to each of the growth curves using the non-linear regression program Ultrafit v3.0.5 (Biosoft, Cambridge, UK) to estimate the cardinal values for temperature (Table 5.7). If growth rate was unaffected by the temperature history of the inoculum, these notional cardinal values should be similar.

Table 5.7: Cardinal temperature values for E coli SB1 in Nutrient Broth

Data Set	Tmin	Tmax
■ 44.4 (±0.1°C) inoculum	4.9 (±1.1)	47.4 (±1.4)
● 10.0 (±0.1°C) inoculum	2.3 (±1.4)	48.3 (±1.0)

Data from the temperature shift experiments for *E. coli* SB1 were also plotted as an Arrhenius plot (Fig. 5.29).

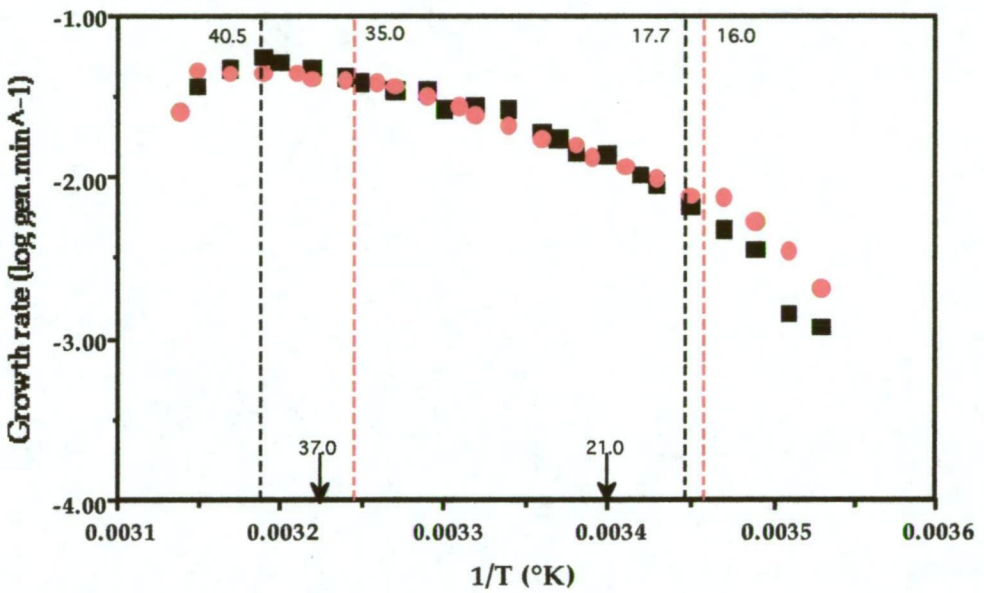

Figure 5.29: Arrhenius plot and boundaries of the normal physiological range for *E. coli* SB1 in Nutrient Broth determined by turbidimetry, where: ■ = 44.4 (± 0.1°C) inoculum with boundaries marked (---), and ● = 10.0 (± 0.1°C) inoculum with boundaries marked (---). Boundaries of the normal physiological range for *E. coli* B/r from Ingraham and Marr (1996) are marked (↑). Some datum points are marked with degrees Celsius.

As there was slight curvature in the region where growth rate is expected to be linear with 1/T, the normal physiological range could not be estimated 'by eye'. Therefore, the data presented in Fig. 5.28 was fitted to the mechanistic model of Ross (1997), which estimates rate limiting activation energy values and defines where deviation from linear Arrhenius kinetics occurs, to determine objectively the boundaries of the normal physiological range. The normal physiological range for the

44.4°C inoculum (40.5°C) was higher than the 10.0°C inoculum (35.0°C), however the lower boundaries for both plots were similar, i.e. 17.7 and 16.0°C respectively.

Effect on RLT of *E. coli* SB1

For *E. coli* SB1 the RLT response for a temperature upshift was different to that for a temperature downshift (Fig. 5.30). For an upshift, RLT showed little variation across the temperature range tested, and remained <0.71. For a temperature downshift, RLT increased in a curvilinear manner with decreasing temperature, reaching a level of 2.5.

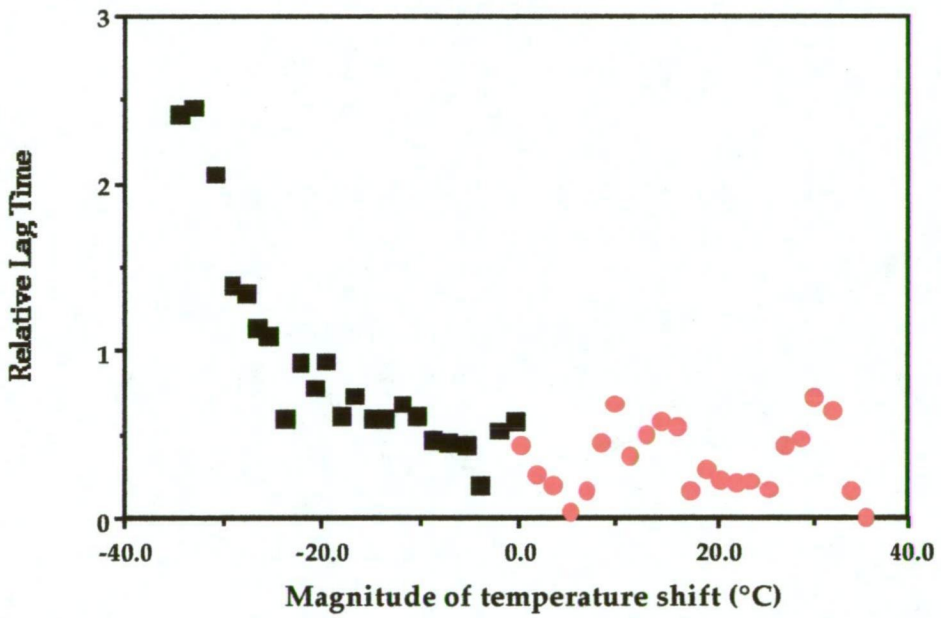

Figure 5.30: Effect of temperature shifts on the relative lag time of *E. coli* SB1 in Nutrient Broth determined by turbidimetry, where: ■ = 44.4 (± 0.1°C) inoculum and ● = 10.0 (± 0.1°C) inoculum.

Effect on lag rate of *E. coli* SB1

For *E. coli* SB1 the lag rate response for a temperature upshift was different to that for a temperature downshift (Fig. 5.31).

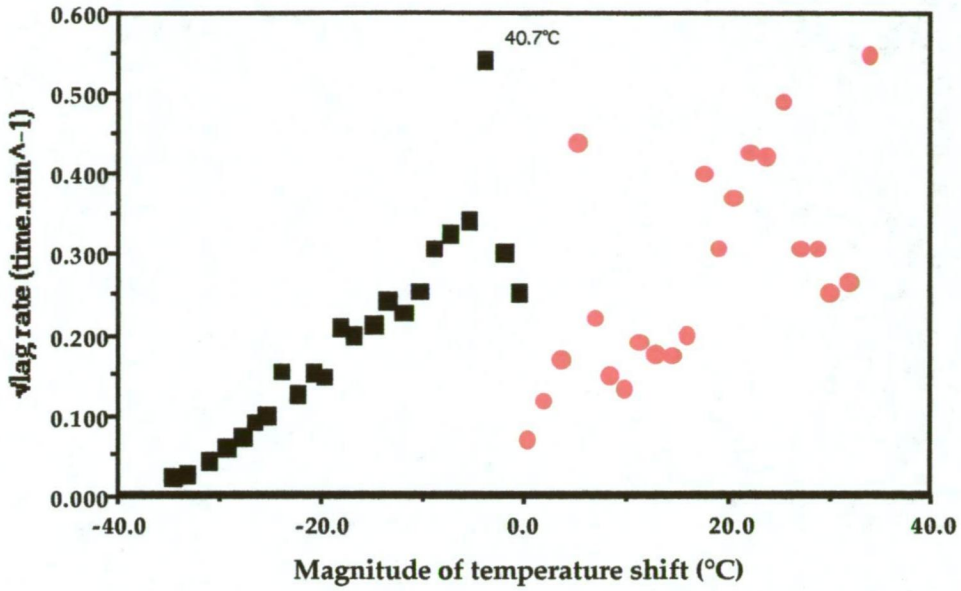

Figure 5.31: Effect of temperature shifts on the lag rate of *E. coli* SB1 in Nutrient Broth determined by turbidimetry, where: ■ = 44.4 (± 0.1°C) inoculum and ● = 10.0 (± 0.1°C) inoculum.

For a temperature upshift, the lag rate response was highly variable. For a temperature downshift, a linear response (y = - 0.10737 + 1.1075e-2x, R^2 = 0.973) was observed below the optimum for growth (approximately 40°C), with lag rate decreasing with decreasing temperature. T_{min} was estimated to be 10°C. For a temperature upshift there was no significant linear relationship (y = 5.5617e-2 + 8.2506e-3x, R^2 = 0.373).

5.3.2.2 Effect of a temperature shifts on *K. oxytoca*

Effect on growth rate of *K. oxytoca*

Temperature shifts were in the range of ΔT= -19.6 to +19.7 °C (*see* S. 5.2.2.2). Raw experimental data are presented in A. 9.4.19. A typical square root plot of growth rate in response to temperature was generated (results not presented), and the same data were plotted as an Arrhenius plot (Fig. 5.32).

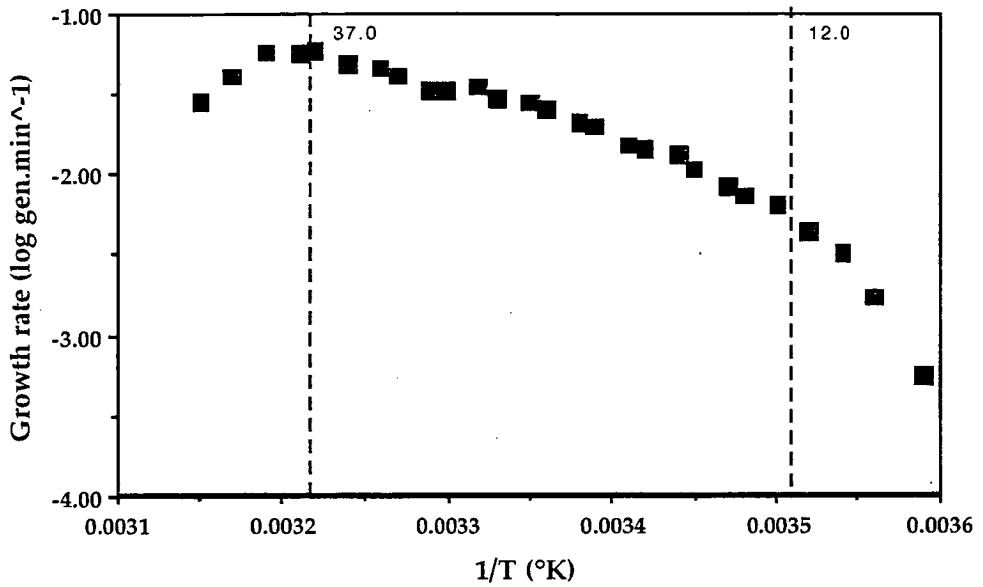

Figure 5.32: Arrhenius plot for *K. oxytoca* (■) in Brain Heart Infusion Broth determined by turbidimetry. Boundaries of the normal physiological range are marked (---). Some datum points are marked with degrees Celsius.

As for the data presented in Figure 5.29, there was slight curvature in the region where growth rate is expected to be linear with 1/T, and the normal physiological range could not be estimated 'by eye'. Therefore, the data presented in Figure 5.32 were fitted to the mechanistic model of Ross (1997) to determine objectively the boundaries of the normal physiological range. The normal physiological range for *K. oxytoca* was estimated to extend from 12.0 to 37.0°C.

Effect on RLT of *K. oxytoca*

For *K. oxytoca* the RLT showed little variation in response either for temperature upshift or downshift across the temperature range tested, and remained <0.71 (Fig. 5.33). Shifts from within the estimated normal physiological range to temperatures just outside this range did not affect RLT.

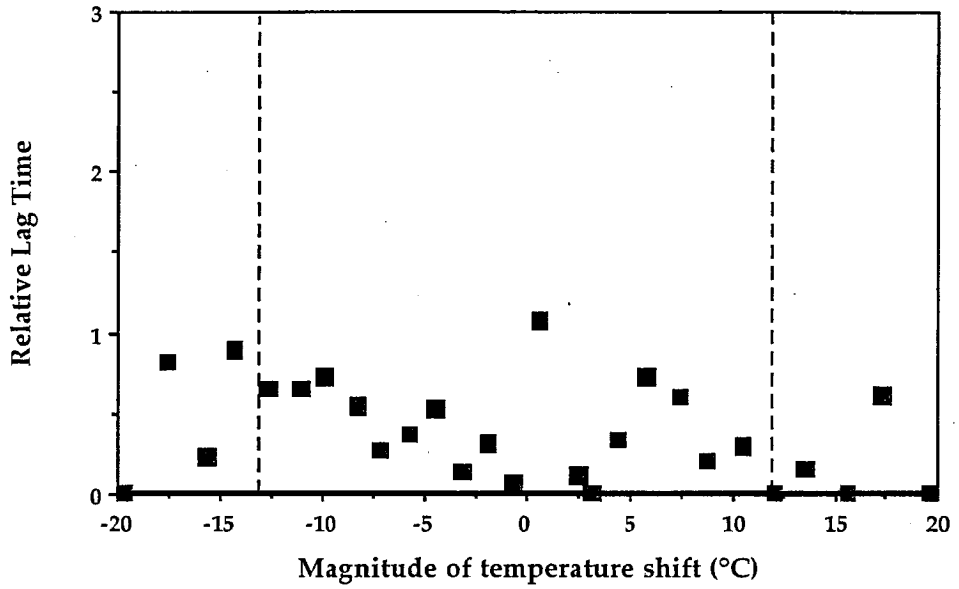

Figure 5.33: Effect of a temperature shift from 25°C in Brain Heart Infusion Broth on the relative lag time of *K. oxytoca* (■) determined by turbidimetry. The estimated normal physiological range is marked (---).

Effect on lag rate of *K. oxytoca*

For a *K. oxytoca* culture subjected to a temperature downshift from within the normal physiological range, the lag rate decreased as temperature decreased (Fig. 5.34). The relationship between lag rate and temperature for a temperature upshift or downshift was irregular. Using linear regression for data below the optimum for growth, estimated to be around 37°C from a plot of square-root of growth rate versus temperature (not shown), T_{min} was estimated to be 8.0°C for the temperature downshift data ($y = - 0.19232 + 2.3786e\text{-}2x$, $R^2 = 0.614$). For a temperature upshift there was no significant linear relationship ($y = - 8.2760e\text{-}2 + 1.3644e\text{-}2x$, $R^2 = 0.125$) and T_{min} was estimated at 6.0°C.

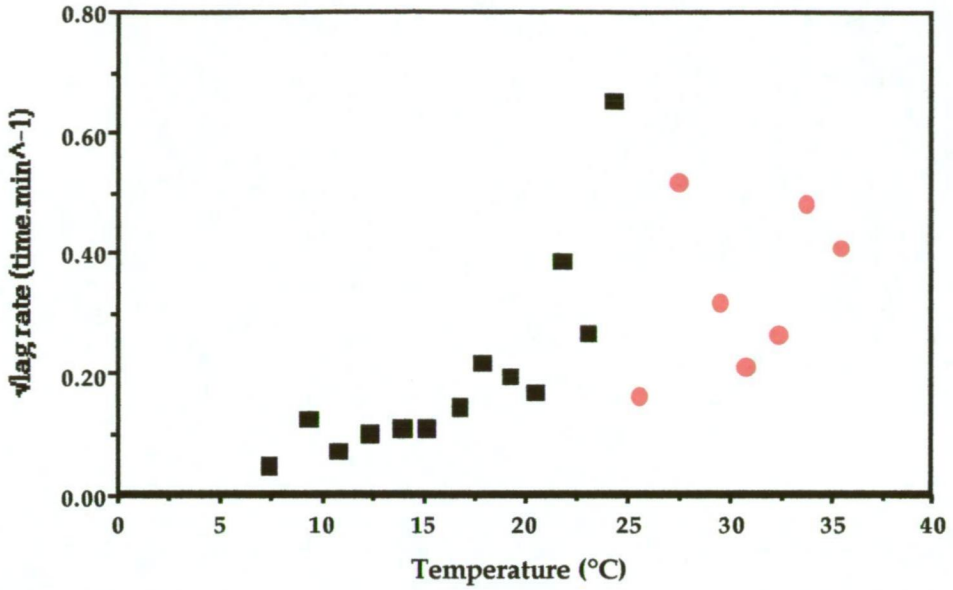

Figure 5.34: Effect of a temperature shift from 25°C in Brain Heart Infusion Broth on the lag rate of *K. oxytoca* for temperatures below 37°C. The data are divided into points equating to a temperature downshift (■) and upshift (●).

5.3.3 pH Shift-*E. coli* M23 in complex laboratory media

In general, RLT was extended by increasingly large shifts in pH for a downshift from neutral, i.e. 7.3 (Fig. 5.35). However, RLT appears relatively unaffected until a downshift of > 1.5 pH units (equivalent to pH 6.1). RLT for an upshift appears unaffected, however there are only four data available and the shift is <1 pH unit. While overall the response for a pH downshift appears similar to that for osmotic downshifts, i.e. RLTs are extended, the degree of the RLT is much smaller for pH, with a maximum of 2.5. Raw data are presented in A 9.4.20.

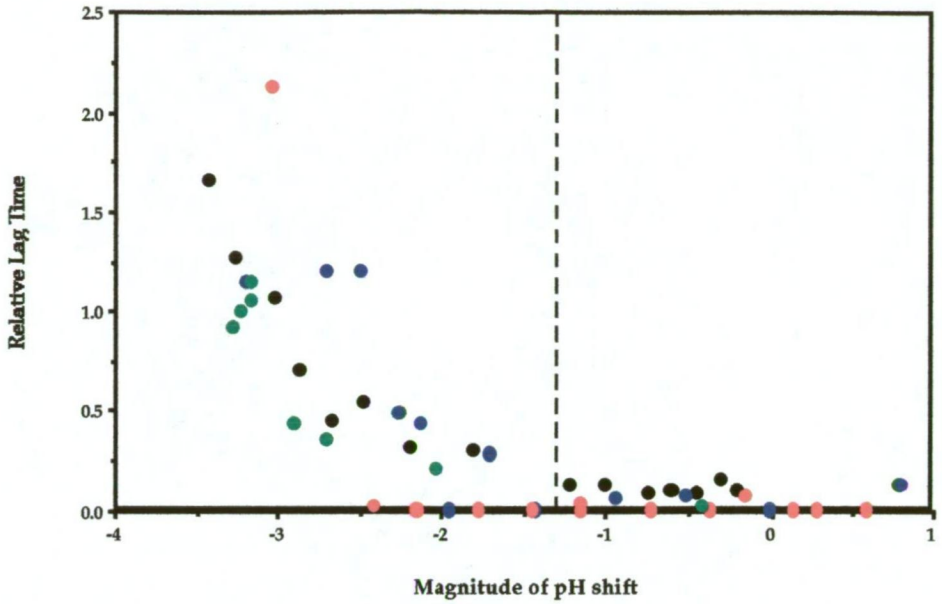

Figure 5.35: Effect of pH shift from 7.3 on the relative lag time response of *E. coli* M23 in Nutrient Broth for four separate experiments. The lower boundary for the normal physiological range for pH is marked (---)

5.3.4 Distributions of RLT

The RLT distribution for all osmotic, pH and temperature shift data described in this chapter is presented in Figure 5.36. A peak in the distribution range occurs at 1.0 RLT. Most RLT values are ≤ 2, and the distribution has a long right hand tail. RLTs greater than 7 are uncommon, and are attributable to viable count data for osmotic downshifts.

The RLT distribution for osmotic, pH and temperature data as individual categories is presented in Figure 5.37. A peak in the distribution range occurred for 1.0 RLT for each data set confirming that observed in Figure 5.35. Temperature and pH RLTs were all <5.

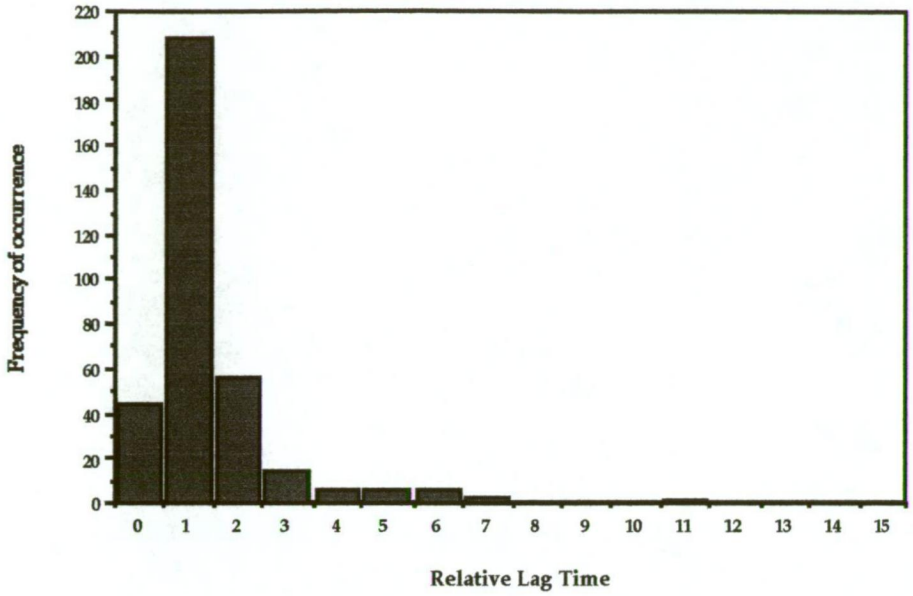

Figure 5.36: RLT distribution of all data for growth in complex and minimal laboratory media.

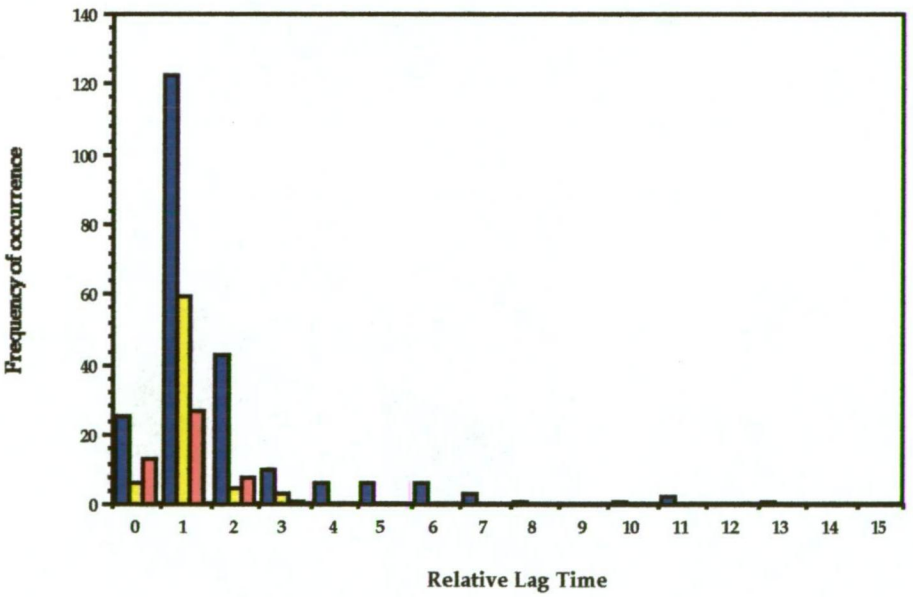

Figure 5.37: RLT distribution for all data for growth in complex and minimal laboratory media at various a_w (■), temperature (■) and pH (■).

5.4 DISCUSSION

Lag times are considered an uncontrollable variable in food microbiology because bacterial contaminants usually have an unknown history. A better understanding of the determinants of lag time and the relative importance of physiological state and environmental conditions would help define the accuracy limits of predictive models (Robinson *et al.*, 1998). Additionally, such knowledge may suggest ways in which the lag phase can be extended to reduce the probability and/or extent of microbial growth in foods. Characterisation of the lag phase and an understanding of which factors induce and affect it may allow more control of this variable.

The relative lag time concept was introduced in S. 5.1.3 as a means of examining the relationship between growth rate and lag time from which a measure of the *amount* of work to be done by the cell before growth is initiated in a new environment can be inferred. If lag time responded to an environmental shift in the same manner as generation time, then we should expect plots of generation time versus lag time to yield straight lines as long as no extra *work* was required. Similarly, if RLT were plotted against a scale reflecting the magnitude of the change in environment a horizontal straight line relationship would be expected if no extra *work* was required.

The objectives of the lag time studies were to:

- test the hypothesis that lag times can be understood in terms of the *amount* of work to be done in adjusting to a new environment and the *rate* at which that work is done,
- characterise the relative lag time response to abrupt osmotic, pH and temperature shifts for a variety of foodborne bacteria,
- test the hypothesis that RLT can be extended by exposing bacterial populations to increasingly harsh conditions
- examine the effect of media type, diluent and temperature of incubation on the recovery of osmotically injured cells, and thus the effect on lag time estimations

- test the hypothesis of Ross (1999) that there is a maximum relative lag time (i.e. the number of generation times under the same conditions) for any viable bacterial population.

In addition to the above objectives, the concept of a normal physiological range for a_w was investigated.

5.4.1.1 Osmotic shift

When a culture was shifted to a new osmotic environment, growth rate resumed at the rate expected in the new environment (Figures 5.1 to 5.4). To reinforce this observation statistically, linear regression was used to calculate a_{wmin}. The resultant estimates were not true indicators of a_{wmin} as only a portion of the growth curve was analysed. Rather they were intended to test the hypothesis that each growth rate curve, despite its different inoculum history, responded similarly. The results presented in Tables 5.1 to 5.4 support this hypothesis. Although linear regression analysis of the *E. coli* data revealed that the *x* intercepts, and hence a_{wmin}, were significantly different, it should be noted that the values for a_{wmin} were generally within the measurable limits for the a_w meter ($\pm$ 0.003 a_w units).

Bacteria respond universally to the temporary loss of turgor following a hyperosmotic shock. Compatible solutes are accumulated, either by transport, synthesis or a combination of both, to increase internal osmotic pressure and therefore restore turgor. However, the type of compatible solute and the mechanisms employed for uptake or synthesis differ between genera and species (*see* S. 1.3.2).

Gram-negative bacteria respond to hyperosmotic stress by an integrated two-phase process; accumulation of K^+ is the primary response (Epstein, 1986), followed by the activation of enzymes, transport systems and gene expression for the accumulation of compatible solutes (Booth *et al.*, 1988). For *E. coli* and *S.* Typhimurium, the physiological response to hyperosmotic shock is largely governed by a range of inducible mechanisms. Implementation of these mechanisms requires *work* to be done and takes time. This is reflected in the RLT response observed when

unstressed cells are transferred to increasingly osmotically stressful environments. Generally, the greater the hyperosmotic shock the larger the RLT, i.e. longer lag times are induced as the *amount* of work to be done to adjust to the new osmotic environment increases. Similarly, the *rate* at which the work is done decreases (i.e. generation times are longer). The viable count data gathered for *K. oxytoca* supports the validity of the data gathered turbidimetrically (Fig. 5.8), i.e. a similar RLT responses for a a_w downshift. The disparity in the scale of the RLT estimates may be attributable partly to differences in lag time estimation due to the effect of inoculum size on lag time duration (*see* S. 5.1.2), and to the systematic deviation in estimation of generation times for turbidimetric and viable count techniques (*see* S. 3.1.3).

Interestingly, the RLT relationship for gram-negative organisms in response to hyperosmotic shifts was not strictly linear, thus inferring that the rate of the response to the new environment is important. When RLT was plotted against the magnitude of the a_w shift for two strains of *E. coli* and *K. oxytoca* in complex or minimal laboratory media, and *S.* Typhimurium in complex media, there appeared to be three distinct regions. This pattern was clearest for the *K. oxytoca* data, Fig. 5.8, as there were more datum points in that experiment than for *S.* Typhimurium and the two *E. coli* strains. Typically, the first part of the plot shows relatively little change in RLT, followed by a linear increase until a point where RLT then decreases. It is possible that the initial portion of the RLT response, i.e. approximately between 0 and 2, may indicate the limit of resolution for RLT determinations. It is difficult to distinguish the effects of true, physiologically-based, responses from 'noise' due to the lack of sensitivity of measurement in this range, characterised by relatively fast growth rates and small lag times, each of which has its own measurement error. Examination of the raw data in A. 9.4.9 to A.9.4.12 and A. 9.4.14 reveals that the differences in generation time estimates for most of the strains are small at the conditions near the optimum a_w for growth. In comparison, the corresponding portion of the plots of lag rate (the reciprocal of lag time) for all three strains indicate that the rate of the response decreases linearly or curvilinearly. If the RLT plot for a a_w downshift for *K. oxytoca* in BHI is plotted on a larger scale (not shown) then the first portion of the

graph is not as horizontal as depicted in Fig. 5.8, but increases steadily as that observed for *S.* Typhimurium (Fig. 5.10.). The rate of this increase in RLT however, is slower than for the second portion depicted in the plot. Thus it would appear that even if the change in osmotic environment is small, there may be an *amount* of work to be done. More data collected by viable count method would assist in characterising this part of the RLT plot and determining whether there is a lower limit of resolution for RLT.

From the RLT plots (Figures 5.6 to 5.11) it is readily apparent that longer lag times are induced by a a_w downshift thus, as indicated above, the *amount* of work increases but the *rate* at which the work is done decreases (reflected in the longer generation times). However, this corollary is not true for the entire RLT plot. A point is reached where the RLT begins to decrease, the third portion of the RLT plot. Where the RLT plot "turns over" appears to occur at a similar point for all three strains, corresponding to a a_w of around 0.960 and a shift of ~0.035 a_w units. It appears that there is comparatively less *work* to be done after a shift of this magnitude. An explanation for this third portion of the RLT plot is examined in Chapter 6.

It would be expected that once the mechanisms in response to hyperosmotic shock are activated they become a part of the cells' normal functioning, i.e. they are osmotically habituated. If the cell is grown at a reduced a_w these mechanisms are already in operation and do not need to be initiated again, thus shifting to more favourable conditions will result in shorter lag times. The *rate* of work increases as the osmotic environment becomes more favourable for growth (i.e. growth rates are faster, thus generation times are shorter). From the RLT plots, RLT increases slightly as the new osmotic environment becomes more favourable for growth. Therefore, there is still some additional *work* to be done to adjust to the more favourable osmotic conditions, however it is comparatively less *work* than that required for a a_w downshift of equal magnitude. A similar RLT response was elicited when cells grown at an intermediate a_w were shifted to a more favourable osmotic environment. However, when cells grown at intermediate a_w were shifted to less favourable environments, RLT remained consistently lower. Closer inspection of the raw data for the

intermediate shifts, presented in A. 9.4.9 to A. 9.4.11, reveals that lag times were short, i.e. <30 minutes[2]. This observation supports the hypothesis that there is a limit to the sensitivity of the turbidimetric technique under circumstances where lag is resolved quickly, and thus a lower limit of resolution for RLT.

Gram-positive bacteria maintain a higher turgor pressure than gram-negatives and posses constitutive and inducible mechanisms for osmoregulation. Constitutive mechanisms for the accumulation of compatible solutes may play a role when the organism is not osmotically stressed and contribute to the osmotolerance usually displayed by gram positive organisms. *L. monocytogenes* has constitutive transport systems for the accumulation of glycine betaine and carnitine (S. 1.3.2.2.2). *S. aureus* has constitutive transport systems for proline uptake (high- and low-affinity) and uptake of glycine betaine (high-affinity) (S. 1.3.2.2.1). Thus the gram-positive bacteria have mechanisms in place to cope with a a_w downshift.

For *S. aureus* the RLT values remain small (Fig. 5.11), i.e. the *amount* of work to be done to adjust to a harsher environment increases, but relatively more slowly than for the gram-negative strains. A similar response is elicited for *L. monocytogenes* over most of the a_w range tested, however near the growth limits RLT begins to increase, signifying more *work* is required to resolve lag at these a_ws. Closer inspection of the raw data in A. 9.4.16 and A. 9.4.17 reveals short lag time estimates scattered over a small range for all the *S. aureus* data and most of the *L. monocytogenes* data. For the *S. aureus* data in particular there are a number of negative lag time estimates. Again, this supports the hypothesis that there is a lower limit of resolution for RLT.

The osmotic shift experiments support the hypothesis of Robinson *et al.* (1998) that 'the *amount* of work will increase and the *rate* will decrease as conditions in a new environment deviate farther from those under which an inoculum is grown'. However, this is true only for a hyperosmotic

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[2] The data also included a number of negative lag time estimates.

shift, i.e. a a_w downshift. As has been demonstrated, this does not apply to a_w upshifts as the *rate* will increase as the environment becomes more favourable for growth (i.e. growth rates get faster).

5.4.1.2 Defining a normal physiological range for a_w

For temperature, 'cell yield' remains constant for much of the growth permissive range and begins to decline near the temperature limits for growth (Ross, 1997). Krist *et al.* (1997) reported a similar response for a_w, with 'cell yield' remaining high over most of the growth permissive range and declining to zero at the low a_w extreme. The results presented in this thesis support the findings of Krist (1997) (*see* Fig. 5.17). The osmotic history of the inoculum had no effect on 'cell yield'. Ross (1997) noted that the temperature at which 'cell yield' began to decline coincided with temperatures that define the boundaries of the normal physiological range. Similarly the question, 'can a normal physiological range for a_w be defined from the results from 'cell yield' experiments?'.

The normal physiological range for temperature can be identified readily using Arrhenius plots interpreted by mechanistic models for the effect of temperature on microbial growth (McMeekin *et al.*, 1993, Krist *et al.*, 1998b). However, no analogous mechanistic basis has been proposed to describe the bacterial growth rate response to a_w, therefore definition of a normal physiological range in the same way as temperature is not possible.

Effects on lag phase duration for shifts from within the normal physiological temperature range to temperatures below this range have been reported in the literature (*see* S. 5.1.2). In S. 5.2.2. an increase in RLT in response to a temperature downshift was found to coincide with the lower boundary of the normal physiological range for *E. coli* (Fig. 5.30). Thus, experiments designed to investigate a potential correlation between the effect of osmotic shifts on lag phase duration and change in 'cell yield' were conducted. It was anticipated that, as for temperature, the a_w where yield begins to decline may represent the lower boundary of a normal physiological range for a_w.

When 'cell yield' and RLT in response to a_w downshifts of *E. coli* and *K. oxytoca* were plotted together (Figures 5.19 and 5.21) a similar response to that described above was observed , i.e. the a_w at which 'cell yield' began to decline coincided with an increase in the RLT. This may be interpreted as, that beyond this critical a_w, the *amount* of work needed to resolve lag increased. This interpretation is supported by the observation that for *K. oxytoca* a change in lag rate occurred at this critical a_w value (Fig. 5.22). The results for *E. coli* SB1 were less clear (Fig. 5.20). However, it is possible that there were insufficient datum points on the plot to determine where the change in lag rate actually occurred.

In summary, for a temperature downshift, the temperature at which RLT begins to increase coincides with the lower limit of the normal physiological range for temperature of *E. coli* reported in the published literature, and the temperature at which 'cell yield' changes. Similarly, the results in this thesis demonstrate that, for a a_w downshift, the a_w at which 'cell yield' begins to decline correlates with a change in lag phase duration denoted by an increase in RLT. Thus, it is hypothesised that the critical a_w value at which 'cell yield' changes denotes the lower limit of a normal physiological range for a_w. For *E. coli* SB1 this occurred at a_w 0.975, and is in agreement with another literature report for this strain (Krist *et al.*, 1998a). For *K. oxytoca* the value at which 'cell yield' changed was a_w 0.980.

For the food industry, the value in defining the normal physiological range for foodborne bacteria lies in identifying boundaries beyond which bacterial lag times can be extended. Such knowledge may allow manipulation of the food matrix to induce bacterial lags, thus improving the shelf life and safety of the food. The concept of a normal physiological range for temperature is established in the literature (*see* S. 1.3.1), and rapid chilling has long been employed as a means of increasing shelf life. The results in this study support the concept of a normal physiological range for a_w and indicate that that this parameter can also be manipulated to induce bacterial lags.

5.4.1.3 Temperature shift

The normal physiological range for *E. coli* is considered to extend from approximately 20 to 37°C (Ingraham and Marr, 1996). However Krist (1997) and Ross (1997) suggest that the lower boundary is closer to 17°C. The Arrhenius plots for *E. coli* SB1 presented in Figure 5.29 support the findings of Krist (1997) and Ross (1997), with an observed lower boundary of around 17°C. The upper boundaries for *E. coli* SB1 in Fig. 5.29 fall either side of the value proposed by Ingraham and Marr (1996). No information on a normal physiological range for *K. oxytoca* was found in the literature. However, from the results presented in Fig. 5.32 it is estimated to extend from approximately 12.0 to 37.0°C.

Shifting bacterial cultures from a temperature within the normal physiological range for temperature are reported to have little effect on lag phase duration, and growth rates at the new temperature are as expected. The results presented in Fig 5.33 for *K. oxytoca* support the earlier literature reports. There are few datum points for conditions outside the normal physiological range, thus it is not possible to determine if the results for *K. oxytoca* are in accordance with literature reports of transitional growth rates for shifts between the normal physiological range and either the high or low range. The results for *E. coli* SB1, however, indicate that if a late exponential culture is grown at a low temperature, an immediate shift to a higher temperature has little effect on lag. This is reflected in the RLT plot (Fig. 5.30) which shows small RLT values, and in Fig. 5.31 which shows no systematic increase or decrease in the lag rate. In contrast, shifting from a temperature above the normal range can affect the lag phase. This is not unexpected as growth at low temperature requires *work* to be done, namely synthesis of proteins and membrane lipids specifically associated with low temperature (Herendeen *et al.*, 1979; Russell *et al.*, 1995). However, the magnitude of the shift is important. Effects on the lag phase are most noticeable when the culture is shifted to temperatures below ~20°C, coinciding with the lower limit of the normal physiological range reported by Ingraham and Marr (1996). RLTs for shifts beyond this temperature then increase systematically as the *amount* of work to be done increases.

With regard to temperature, it has been reported that lag phase duration is inversely proportional to the maximum specific growth rate, and that square-root models fitted to both types of data give similar estimates of T_{min} (*see* S. 5.1.2). An inverse relationship between lag phase and maximum specific growth rate was observed for a late exponential phase culture of *E. coli* SB1 shifted from 44.4°C to a range of lower temperatures (Fig. 5.31). However, the T_{min} value estimated from the lag data, 8°C, was higher than that estimated from the growth data, 4.9°C (Table 5.7). The higher T_{min} estimate from the lag rate data is not unexpected as RLT has increased (Fig. 5.30), i.e. lags are longer. For exponential phase *K. oxytoca* shifted from 25.0°C to a range of lower temperatures (Fig. 5.34), the relationship between lag rate and temperature is linear. The T_{min} value estimated from the lag data, 8°C, is much higher than that predicted by a growth rate model for this organism, 0.5°C (Table 3.3). In this instance the higher T_{min} estimate is not related to an increase in RLT (Fig. 5.33).

From the results for cultures subjected to temperature upshifts from a temperature either below (Fig. 5.31) or within (Fig. 5.34) the normal physiological range, the inverse relationship between lag phase duration and growth rate did not hold. This also is not unexpected as the literature reports are for stationary phase cells. As mentioned in S. 5.1.2, the physiological state of the inoculum affects lag time duration. Stationary phase cells are in a quiescent state and require some adjustment, and hence lag times, before exponential growth can be initiated in a new environment. Similarly, for the exponential phase cells subjected to a temperature downshift, work had to be done to resolve lag before exponential growth could re-commence in the new environment. In contrast, comparatively less work is required for cells subjected to temperature upshifts. Thus the relationship between temperature and lag rate for cells subjected to upshifts is less closely correlated.

5.4.1.4 pH shifts

In general, as pH is decreased, RLT increases. The effect is more pronounced for shifts greater than 1.5 pH units. This coincides with the lower limit for the normal physiological range for pH, 6.0 (Ingraham and Marr, 1996), and is close to the lower limit where *E. coli* tolerates changes

in pH_i, 6.5 (Booth, 1985; Booth and Kroll 1989). Close to the optimal pH value *E. coli* is not stressed and pH homeostasis is maintained. As the acidic or alkaline limits are approached, homeostatic mechanisms may break down, leading to changes in growth rate and lag time. Longer lag times are induced as the *amount* of work to maintain pH_i increases but the *rate* at which the work is done decreases. The maximum RLT observed was much smaller than comparable data for osmotic and temperature shifts. However, it should be noted, that unlike the temperature and osmotic shifts described previously, the pH shifts were conducted with a stationary phase inoculum. Physiological history affects lag time duration (S. 5.1.2 and S. 6.3.1) and this may account in part for the small magnitude of the RLT response.

5.4.1.5 Lag time estimates for populations containing injured cells

The presence of sublethally injured cells in a bacterial population has important ramifications for the food industry as viable count data is often used to monitor process hygiene or to define parameters for anti-microbial treatments. Mackey *et al.* (1994) demonstrated the importance of the choice of recovery conditions with their work on heat treated *L. monocytogenes*. They reported that the time necessary to achieve a 5 log reduction in viable numbers was apparently increased by a factor of 1.5 when survivor curves were based on counts on Blood Agar incubated at 25°C rather than on TSA incubated at 37°C.

The presence of sub-lethally injured cells is relevant to the meat industry as carcass processing exposes microbial populations to a variety of stresses, e.g. chilling is known to affect a_w and temperature at the carcass surface. Carcass decontamination strategies such as steam vacuuming or spraying with hot water and organic acids may also sub-lethally injure cells. As a_w is a focus of this thesis, the potential effects of incubation temperature, diluent and recovery media for osmotically stressed cells were investigated. Effects on lag and generation time estimates were also examined. The results are shown in Figures 5.26 and 5.27, and Tables 5.5 and 5.6.

The addition of NaCl to the diluent and plating medium used for viable counts of *K. oxytoca* grown at a_w 0.962 did not enhance the recovery of cells in comparison to diluent and plating media containing no additional salt. Thus it is unlikely that diluent and media confer additional osmotic shocks.

Generation time estimates for *K. oxytoca* grown at a_w 0.962 were unaffected by media type, diluent or temperature of incubation (25 or 37°C). Lag time estimates of osmotically stressed cells *appeared* to be affected, as were, thus, the RLT values. RLT values for counts on the selective medium, MAC, were larger than the non selective media and estimates on the enhanced resuscitation medium, BHAP, were smallest. However, by visual examination of the plots in Figures 5.26 and 5.27, it appears that lag is resolved at around the same time regardless of the recovery medium, temperature or diluent. Mackey and Derrick (1982a) reported that for heat injured *S.* Typhimurium, resistance to a variety of selective media was regained by the end of the lag preceding cell multiplication. Considering their observations, it is possible that the apparent differences in RLT are due to normal experimental variability and insufficient data collected for the entire growth curve. In both experiments the growth curves were not followed into stationary phase, thus the generation and lag time estimates could be improved. It would also appear that the NaCl modified diluent, PWS, reduced the variability in RLT estimates. It is, however, more likely that the reduced deviation in estimates for RLT with PWS diluent is due to the greater amount of experimental control for the viable count data in this experiment. Experience gained from the previous experiment in PW diluent resulted in a reduced number of dilutions plated out at each sample time and there were fewer media tested. Further work, with more viable count data collected in the early stages of growth, is required to determine if, as has been reported for heat injured cells, lag time estimates for osmotically injured cells are not affected by the choice of recovery medium.

The report of Mackey and Derrick (1982a) and the results presented here indicate that it would be prudent to include pyruvate in recovery media for sub-lethally injured cells as a precaution. The smallest lag times, and thus RLT value, for both experiments was recorded for BHAP. Although

experimental variability is proposed to account for the disparity in lag time estimates, a 'fail-safe' policy is usually adopted in predictive modelling, thus there is value in utilising the shortest lag time estimate.

The observations presented above confirm that the injury observed by viable count determinations for *K. oxytoca* after an abrupt a_w downshift is a genuine reflection of loss of culturability as opposed to an artifact of diluent, media type and temperature of incubation.

5.4.1.6 Lag time limits

The results from the lag time studies presented here demonstrate that lag times are highly variable. This poses problems for their inclusion in predictive models. However, this apparent variability can be reduced by using the concept of relative lag times (RLT), or generation time equivalents. From a collation of literature data, Ross (1999) suggested that, although lag times may take almost any value, there is a common pattern of distribution of relative lag times for a wide range of species across a wide range of conditions. He hypothesised that there might be an upper limit to the length of relative lag times and reported the distribution of RLTs has a common pattern for many organisms and has a sharp peak in the range of 4 to 6 generation time equivalents, and an upper 95th percentile in the range of 10 to 15 generation time equivalents.

The results presented here are in accord with Ross's analyses of data obtained from the literature and data actively gathered by novel experiments. The observed RLT for a variety of foodborne bacteria and conditions was usually less than 7 and always less than 12, even for extreme shifts (Figures 5.36 and 5.37). A sharp peak for RLT values of 1.0 is slightly lower than that observed by Ross (1999). In comparison to Ross's data, which contained significant numbers of viable count data, the RLT data presented here is predominantly turbidimetric. Turbidimetric data may generate smaller RLT values than viable count due to higher inoculum levels resulting in shorter lag phases. Some larger RLT values were generated by viable count data, suggesting that inclusion of more viable count data may shift this peak further to the right.

5.4.1.7 Summary

It is evident that lag times are highly variable. This apparent variability can be reduced by using the concept of relative lag times or "generation time equivalents", i.e. the ratio of lag time to generation time (RLT). Ross (1999) reported a common pattern of distribution of relative lag times for a wide range of species across a wide range of conditions, and the data presented here share that same pattern.

The results from the a_w shift experiments and temperature shift experiments support the hypothesis that lag time can be understood in terms of the *amount* of work to be done and the *rate* at which that work is done.

For a a_w downshift, differences were observed in the RLT response of the gram-negative and -positive strains tested. In general, RLTs were extended for the gram-negative organisms as conditions became less favourable for growth. In comparison, RLT remained relatively unaffected for the gram-positive organisms. These results were expected on the basis of the strategies for osmoregulation employed by different bacteria.

The RLT response for a a_w upshift differed to that of a a_w downshift for the gram-negative organisms tested. The reduction in RLT at very low a_w was unexpected.

With regard to temperature, the results of this study support previous reports that the square root of lag time is inversely proportional to the maximum specific growth rate. However, this relationship holds only for shifts requiring *work* to be done to resolve the lag phase. In contrast, the relationship for a_w and lag rate is less closely correlated and as such is unlikely to be modelled in the same manner as temperature.

It is hypothesised that the critical a_w value at which 'cell yield' changes denotes the lower limit of a normal physiological range for a_w.

From the lag time studies, the results indicated that the sensitivity of turbidimetric methods to estimate short lag times was doubtful. It is probable that there is a lower limit of resolution for RLT determined turbidimetrically.

6. FACTORS AFFECTING RLT ESTIMATES

6.1 INTRODUCTION

The data presented in Chapters 5 supports the concept that lag phase duration can be understood in terms of 'amount' of work and work 'rate', and that RLT can be used to characterise lag phase duration in response to abrupt environmental shifts. RLT has been suggested as a useful tool allowing inclusion of lag time estimates into predictive models and risk assessments. Information on the distributions of RLTs is certainly an important contribution (S. 5.3.4).

As RLT is a ratio, changes in either generation time or lag time can affect its' value. Two areas have been identified that may affect the reliability of generation and lag time estimates, and hence RLT; the physiological history of the inoculum and irregularly shaped growth curves due to either experimental error or changes in growth patterns in some environments.

6.1.1 Physiological history of inocula

Bacteria have evolved adaptive responses to face the challenges of changing environments and to survive under conditions of stress (Abee and Wouters, 1999). As part of the adaptive process growth may cease, i.e. a lag phase, as the bacteria seek to regain equilibrium with their environment through structural and functional alterations to the cell. In general, overall protein synthesis is inhibited and 'stress'/'shock' response proteins are synthesised. While some proteins are induced under many different conditions (e.g. universal stress proteins), others are induced only in response to a specific stress (Bearson *et al.*, 1997). Perhaps the best characterised adaptive response in *E. coli* is the transient induction of several proteins in response to heat-shock (for a review *see* Gross, 1996). Shock proteins have also been studied in *E. coli* in response to other environmental stresses including low temperature (Goldstein *et al.*, 1990; Jones *et al.*, 1987), pH (Heyde and Portalier, 1990; Hickey and Hirshfield, 1990), osmotic stress (Clark and Parker, 1984; Botsford, 1990; Jenkins *et al.*, 1990) and starvation (Groat *et al.*, 1986; Kolter, 1992).

It is well recognised that exponential phase cells are more sensitive to inimical processes than stationary phase cells. This difference in resistance to processes such as heating, freezing, biocides and antibiotics is currently explained by the differential expression of biosynthetic pathways, gene regulators and associated enzyme systems that provide an adaptive advantage to the stationary phase cell (Rees *et al.*, 1995; Dodd *et al.*, 1997). Although not considered differentiated, stationary phase cells of enteric bacteria have many properties in common with spores, such as the ability to survive prolonged periods of starvation and a strong multiple-stress resistance (Hengge-Aronis, 1996).

Typically in industry the physiological history of cells is unknown and a worst-case strategy is often adopted when making assumptions about microbial growth in foods. The physiological history of the cell is considered to have an effect on lag times (McMeekin *et al.*, 1993; Baranyi and Roberts, 1994), yet examination of the literature reveals little hard data to support this claim.

6.1.2 Irregular growth curves

In S. 5.1.3. the *amount* of work to be done to resolve lag was considered to involve the additional biosynthetic demands to adjust to the new environment. The RLT plots for temperature and pH downshifts logically exemplify that more work is required to prepare for growth at harsher conditions, i.e. progressively more biosynthetic demands are placed on the cell. It would be reasonable to expect the response to osmotic downshifts to be similar. The RLT response of gram-negative organisms to shifts from optimal a_w to below a_w~0.965 (S. 5.3.1.1) appears anomalous because RLT apparently declines after this point. That there is *less* work to be done after a shift of a certain magnitude seems unlikely. A possible explanation is that a different osmoregulatory mechanism in gram-negative organisms is employed to cope with large and abrupt a_w shifts. However, before investigating the mechanism of a previously unreported phenomenon it was considered prudent to explore other explanations.

Closer examination of both turbidimetric and viable count data used to produce RLT plots for osmotic downshifts, revealed that the growth curves at lower a_w are indicative of cells undergoing diauxic growth. This phenomenon occurs if two energy sources are present in the growth medium simultaneously and the enzyme needed for utilisation of one the energy sources is subject to catabolite repression (Monod, 1949). Growth first occurs preferentially on the more readily catabolisable energy source, followed by a temporary cessation before growth is resumed on the other energy source. This is illustrated in Figure 6.1.

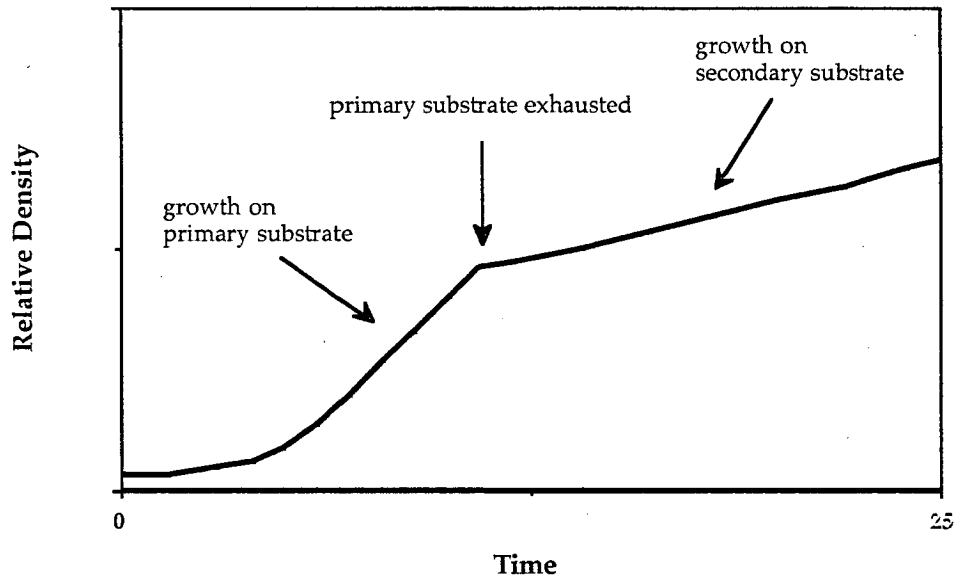

Figure 6.1: Depiction of diauxic growth on a mixture of primary and secondary substrate, after (Brock and Madigan, 1991).

The enzyme responsible for utilisation of the secondary substrate is inducible, but its synthesis is also subject to catabolite repression. Thus while the primary substrate is present in the medium the enzyme is not synthesised. If the primary substrate is exhausted, catabolite repression is abolished, the enzyme is synthesised and growth, at a slower rate, on the secondary substrate occurs. The diauxic shift between the two substrates often involves a small lag period, referred to as 'diauxic lag'.

If substrate limitation occurs before the cells have reached their maximum growth rate the steepest part of the exponential growth phase may not be

observed. This affects both generation and lag time estimates if linear regression is used. A growth curve for stationary phase cells of *S.* Typhimurium inoculated from basal BHI media into BHI at a_w 0.951 is presented in Figure 6.2 and exhibits the diauxic growth pattern.

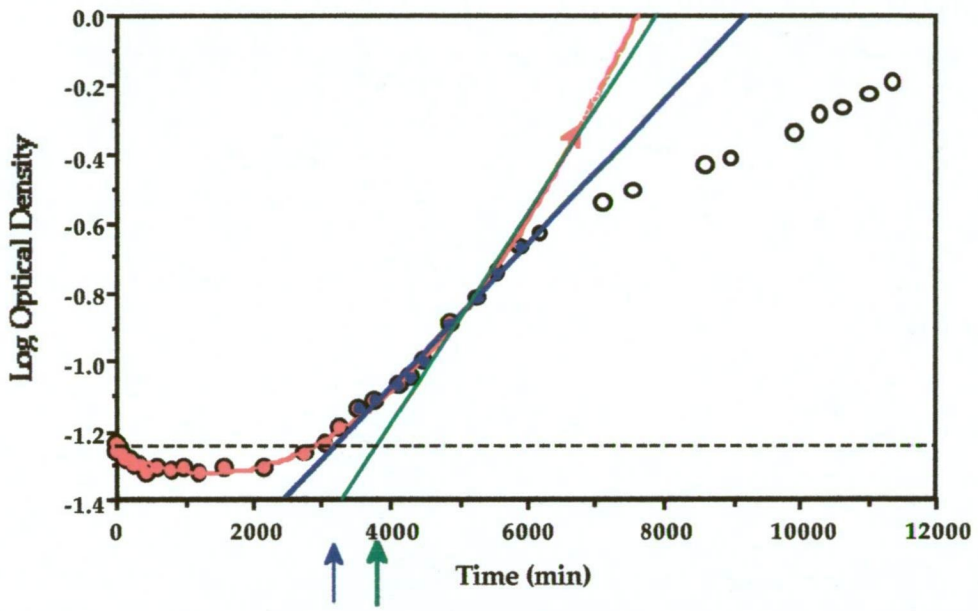

Figure 6.2: Growth of *S.* Typhimurium at a_w 0.951 determined by turbidimetry (○). Points used for estimation of generation and lag time by linear regression are marked ●, and the line of best fit (---). The potential shape of the curve in the presence of the preferred substrate is marked ●, and a hypothetical line of best fit is plotted (---). The starting optical density is plotted (---) and intercepts corresponding to the lag time estimates are marked (↑).

Using the linear regression method described in S. 2.1.3.3. the lag time is 3063.85 minutes (*see* A. 9.4.21). However, it is clear that the steepest part of the exponential phase of the growth curve may not have been observed. Plotting a hypothetical curve to the data and extrapolating the steepest portion, marked in green, provides a faster growth rate estimate and longer lag phase of ~3900 minutes. Underestimating growth rate affects derived RLT estimates.

Both decreasing the generation time and increasing lag time increases the RLT value, compounding the effect of the underestimation of growth rate on the derived RLT estimate.

6.1.3 Objectives

Section 1.2 emphasised that a number of environmental parameters influence the growth, death and survival of microorganisms, and that on the carcass surface, a_w is an important factor in controlling microbial proliferation (S. 1.4.3). This chapter systematically examines the effect of physiological history of the cell on lag time duration in response to a_w downshifts using both turbidimetric and viable count methods. Differences in the lag time responses due to the physiological state of the inoculum may assist in defining upper and lower limits to RLT.

It is hypothesised that the 'downturn' in RLT at low a_w is an artifact, i.e. a nutrient becomes exhausted under conditions of high osmotic stress, low a_w, leading to an alteration in the shape of the growth curve and hence generation and lag time estimates. In consequence, RLT values decrease, i.e. the lag phase is shortened and generation time estimates are longer, leading to the 'downturn' observed in the RLT plots. To test this hypothesis conditions where the preferred substrate does not become limiting were provided to counter diauxic growth and produce curves with more realistic generation and lag time estimations.

Aside from alterations in growth patterns, experimental error can impact on the shape of growth curves. Before using the RLT concept with confidence as a tool to characterise lag times and for application in industry, it would be useful to know how sensitive it is to the estimate of the rate of growth and lag time, and how reproducible RLT is.

6.2 MATERIALS AND METHODS

Although *E. coli* has been the main target of much of the work presented in this thesis, *S.* Typhimurium was used for most of these experiments as it has a wider a_w growth permissive range than *E. coli*, down to a_w 0.945

(Jay *et al.*, 1997). *K. oxytoca*, which has a similar a_w growth permissive range to *S.* Typhimurium, was used in one experiment.

6.2.1 Physiological history

OVERVIEW

The effect of physiological history on lag phase duration of *S.* Typhimurium subjected to a_w downshifts was determined by two turbidimetric and one viable count experiment.

In the first turbidimetric experiment an inoculum of either exponential, stationary phase or a controlled mix (9 to 1 ratio) of culture was prepared in basal BHI broth. The inocula were then dispensed into a series of a_w modified BHI broths, i.e. a_w downshifts only. Growth was monitored turbidimetrically and generation and lag time estimates calculated using linear regression. Relative lag times were also calculated. The initial experiment was repeated for exponential and stationary phase cells, with the series of a_w modified test broths at smaller a_w intervals. The RLT responses were compared to data for late exponential phase *S.* Typhimurium shown in Figure 5.10.

For the viable count experiment, exponential and stationary phase inocula were diluted in BHI then dispensed into a series of three NaCl modified BHI broths at various a_w levels. Growth was monitored by viable counts on BHAP recovery media.

METHOD

6.2.1.1 Inoculum preparation

The late exponential phase inocula described previously (S. 2.1.2.1) comprise a mix of predominantly exponential and some stationary phase cells. Purely exponential phase and stationary phase cultures were required to investigate effects of physiological history on lag phase duration.

6.2.1.1.1 Exponential inoculum

S. Typhimurium was resuscitated from cryogenic storage as described in A. 9.1.3. A loopful of a 24 hour plate culture of *S.* Typhimurium was inoculated into 50mL of pre-warmed BHI in a sterile 125mL stoppered conical flask. This primary broth was incubated in a shaking water bath at 25 (±0.1)°C for 14 hours. 50μL of primary culture was aseptically dispensed into another 50mL of pre-warmed BHI in a sterile 125mL stoppered side-arm flask. This secondary culture was incubated in a shaking water bath at 25 (± 0.1) °C and growth monitored turbidimetrically at 540nm using an analogue spectrophotometer. The secondary culture was incubated until %transmittance fell to 20%. This process was repeated using the secondary culture to provide the exponential phase inoculum.

6.2.1.1.2 Stationary phase inoculum

S. Typhimurium was resuscitated from cryogenic storage as described in A. 9.1.3. A loopful of a 24 hour plate culture was inoculated into 50mL of pre-warmed BHI in a sterile 125mL stoppered conical flask. The broth was incubated in a shaking water bath at 25 (±0.1)°C for 26±0.5 hours. This culture was used as the stationary phase inoculum.

6.2.1.1.3 Controlled 'mix' inocula

Exponential and stationary phase cells were combined in controlled ratios. 9mL exponential and 1mL stationary phase inocula were added to a sterile 30mL McCartney bottle and mixed by vortexing. This inoculum is hereafter referred to as 90% exponential phase. Similarly, 9mL stationary and 1mL exponential phase inocula were added to a sterile 30mL McCartney bottle and mixed by vortexing. This inoculum is hereafter referred to as 90% stationary phase.

6.2.1.1.4 Inocula for viable count experiments with *S.* Typhimurium

For the viable count experiments, exponential and stationary phase inocula were serially diluted in BHI to a concentration of $\sim 10^{5\text{-}6}$ cfu.mL^{-1}.

6.2.1.2 Media preparation

For turbidimetric experiments a series of a_w adjusted broths was prepared as described in S. 2.1.4. BHI (a_w 0.993) and BHI with 80g.L^{-1} NaCl (a_w 0.949) were prepared. The two media were combined in various ratios to generate a series of 12 broths at a range of a_w values. For a second experiment BHI (a_w 0.995) and BHI with 85g.L^{-1} NaCl (a_w 0.940) were prepared. The two media were combined in various ratios to generate a series of 20 broths at a range of a_w values.

For the viable count experiment BHI at a_w 0.976, 0.964, and 0.956 were prepared by adding 40, 55 and 70g.L^{-1} NaCl to basal BHI (a_w 0.995). 50mL of NaCl modified broths were added aseptically to sterile 200mL stoppered conical flasks.

6.2.1.3 Growth curves

For turbidimetric experiments L-tubes were inoculated and growth monitored as described in S. 2.1.3.1.1. To achieve the same starting %T, the amount of inoculum added varied: 0.25mL of exponential and 90% exponential phase and 0.1mL of stationary and 90% stationary phase cells were used.

For viable count experiments 1mL of a 10^{-2} dilution of exponential phase preparation and 1mL of a 10^{-4} dilution of stationary phase preparation was added to each flask and cultures were incubated with shaking at 25.0 (± 0.1)°C. 0.1mL aliquots were removed at regular time intervals from each of the 6 flasks and serially diluted in PW as required. 0.1mL aliquots were surface plated onto BHAP agar using a Spiral Plater (A. 9.2.). Plates were incubated for 24 hours at 35°C.

All growth curves were analysed using linear regression (S. 2.1.3.3) to estimate generation and lag times. RLTs were calculated.

6.2.2 Diauxic growth

OVERVIEW

As the phenomenon of diauxic growth was first described by Monod (1949) for glucose this substrate was provided in excess in the outgrowth medium. As the 'downturn' in RLT is most extreme for purely exponential phase cells, this type of inoculum was used.

Osmotic downshifts in BHI and BHI with 0.6 or 2% added glucose were performed initially for *S.* Typhimurium and growth monitored turbidimetrically. A second experiment studied *S.* Typhimurium and *K. oxytoca* in BHI with 2% added glucose, in a series of a_w modified test broths at narrow a_w intervals in the growth permissive range below a_w ~0.980. RLTs were calculated and compared.

METHOD

6.2.2.1 Inoculum preparation

Exponential phase inocula of *S.* Typhimurium and *K. oxytoca* were prepared as described in S. 6.2.1.1.

6.2.2.2 Media preparation

For each experiment a series of a_w adjusted broths was prepared as described in S. 2.1.4. For the initial *S.* Typhimurium experiments pairs of media were prepared as follows: BHI (a_w 0.994) and BHI with 80g.L^{-1} NaCl (a_w 0.944); BHI with 0.6% glucose added (a_w 0.994) and BHI with 0.6% glucose and 80g.L^{-1} NaCl (a_w 0.941); BHI with 2% glucose added (a_w 0.993) and BHI with 2% glucose and 80g.L^{-1} NaCl (a_w 0.939). The pairs of media were combined in various ratios to generate a series of 10 broths at a range of a_w values for each glucose concentration. For the combined *S.* Typhimurium and *K. oxytoca* experiment, BHI (a_w 0.994) was prepared for growth of the inocula. BHI with 2% glucose and 35g.L^{-1} NaCl (a_w 0.979) and BHI with 2% glucose and 80g.L^{-1} NaCl (a_w 0.947) were prepared then combined in various ratios to generate a series of 20 broths at a range of a_w values.

6.2.2.3 Growth curves

L-tubes were inoculated and turbidimetric growth monitored as described in S. 2.1.3.1.1. All curves were analysed using linear regression (S. 2.1.3.3) to estimate generation and lag times. Growth rate, lag rate and RLTs were calculated.

6.2.3 Reproducibility and variability

6.2.3.1 Reproducibility of RLT

Separate osmotic shift experiments in which inoculum preparation was the same for any strain were identified for a_w downshifts. Duplicate experiments presented in S. 6.2.1 of exponential phase and stationary phase *S.* Typhimurium were identified (Figures 6.1 and 6.2). A second a_w downshift experiment for late exponential phase *E. coli* SB1 was undertaken as described in S. 5.2.1.1. RLT data from separate experiments for each strain were plotted together, and the difference in RLT was also calculated.

6.2.3.2 Simulations

An idealised bacterial growth curve, log numbers *versus* time, was devised. The curve consisted of 7 equidistant, with respect to time, points in total: 3 points represented the lag phase, 3 points the exponential phase and 2 points the stationary phase. The central point in exponential phase was fixed. The slope of the exponential phase was altered by changing the value of the last point in exponential phase by 8 regular increments of 5%. The idealised growth curve and data manipulations are presented in Figure 6.3. The resultant growth curves were used to examine the effect of changes in the slope of the exponential phase on the subsequent lag, and hence RLT, estimations. Linear regression (*see* S. 2.1.3.3) was used to estimate generation and lag times.

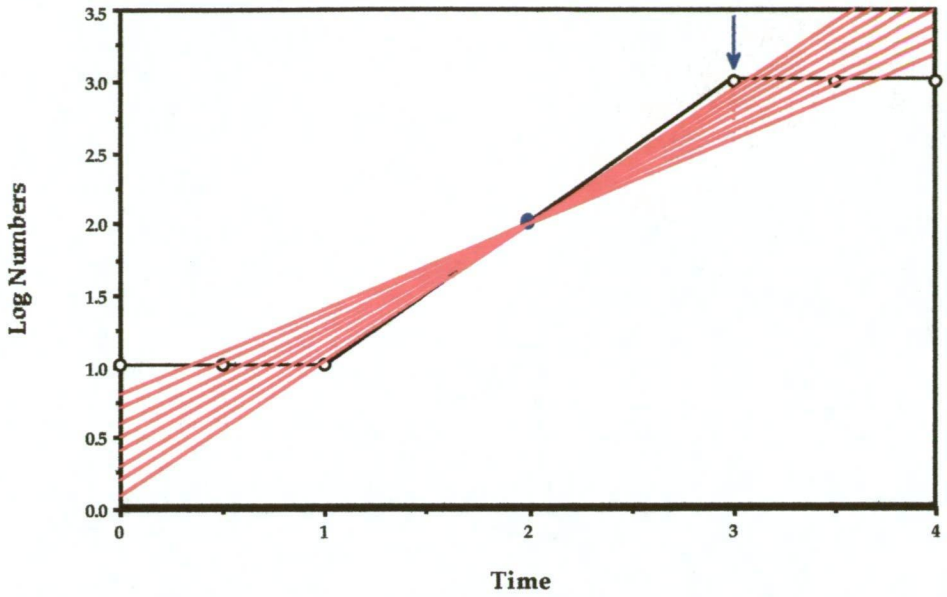

Figure 6.3: Pattern of data used to generate growth curves with different slopes. The central point (●) is fixed and the last point in the exponential phase (↑) was varied to produce 8 different slopes, marked (---).

6.3 RESULTS

Universal scales for *x* and *y* axes were not employed for the following plots to facilitate clear descriptions of patterns of kinetic responses for each bacterial strain.

6.3.1 Physiological history

As described previously for late exponential phase cells, (S. 5.3.1.1), osmotic downshifts increased the RLT response of *S.* Typhimurium, regardless of the physiological history of the inoculum. However, the degree of the RLT response varied with the physiological history of the inoculum. This effect was also observed for viable count data however, the difference in the magnitude of the RLT response for each inoculum preparation was smaller.

6.3.1.1 RLT of exponential, stationary and 'mixed' *S.* Typhimurium

The magnitude of the RLT response of *S.* Typhimurium was greatest for purely exponential phase cells (Figure 6.4). RLT increased as the magnitude of the shift increased to a maximum of ~8 at a_w 0.969. RLT then decreased to ~7. For purely stationary phase cells RLT increased to a maximum of 2 at a_w 0.961 then decreased to 1. The response of the mix containing 90% exponential phase cells was intermediate and the RLT increased to a maximum of 4. The RLT of the mixture containing 90% stationary phase cells was also intermediate but more similar to that of purely stationary phase cells with a maximum RLT of ~2.5. Raw data are presented in A. 9.4.21.

6.3.1.2 Comparison to late exponential phase cultures

The results for a second osmotic downshift experiment conducted with exponential and stationary phase *S.* Typhimurium cells (Fig. 6.5) confirm the observations presented in Figure 6.4. Raw data are presented in A. 9.4.22.

The RLT response for exponential cells increased with the magnitude of the shift, and the maximum RLT value was the same, i.e. ~8. Whilst the stationary phase cells responded in a similar way to the previous experiment, the maximum RLT was higher; ~4 compared with 2. The RLT response for late exponential phase cells (i.e. an unknown mix of predominantly exponential cells and some stationary phase cells) from a previous experiment (Fig. 5.10) was also found to be intermediate.

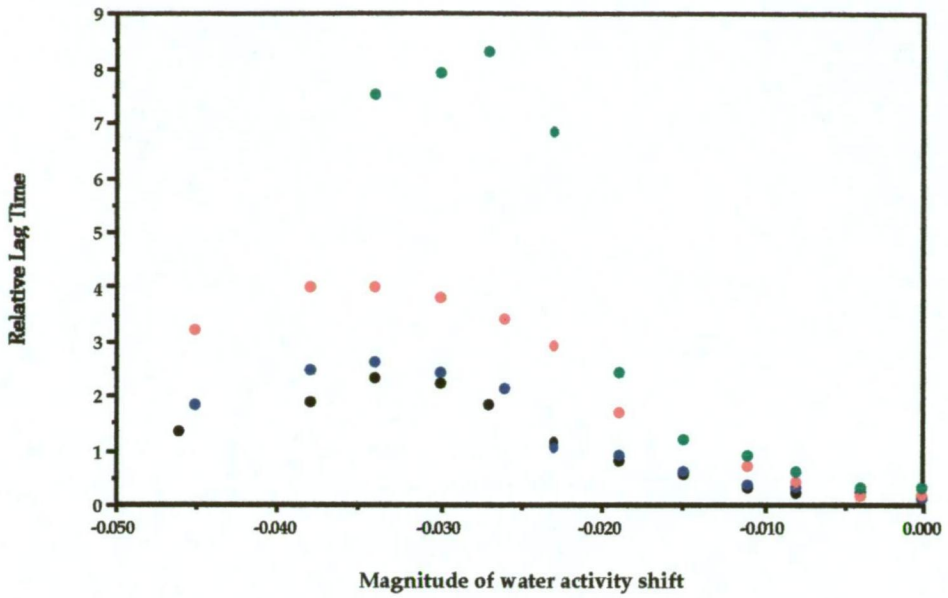

Figure 6.4: Effect of a_w downshift on the relative lag time response of stationary (●), 90% stationary (●), exponential (●) and 90% exponential (●) phase *S.* Typhimurium

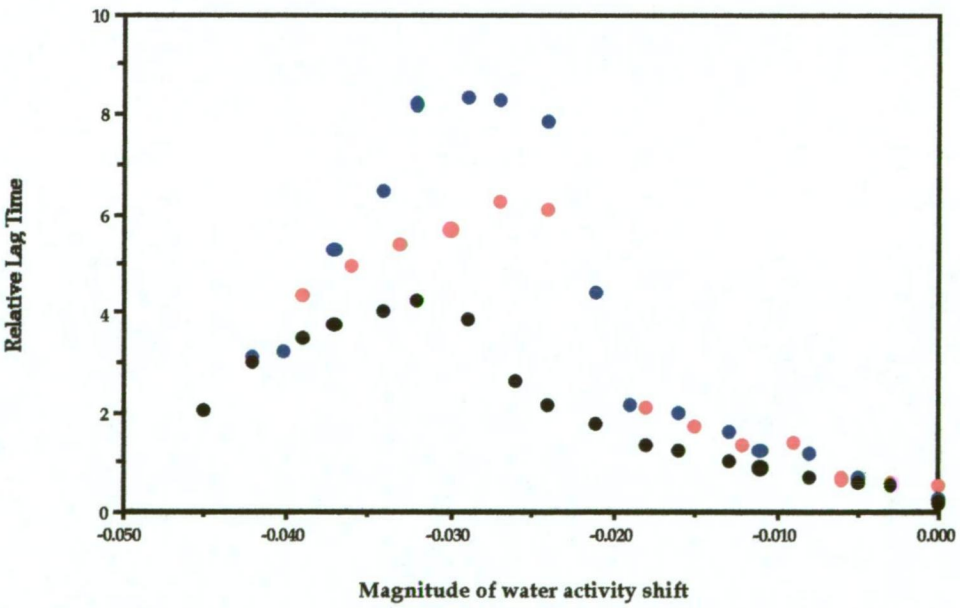

Figure 6.5: Effect of a_w downshift on the relative lag time response of stationary (●), exponential (●), and late exponential (●) phase *S.* Typhimurium

The effect of osmotic downshift on RLT of exponential and stationary phase cultures of *S.* Typhimurium was also determined by viable count. The viable count growth curves for exponential and stationary phase cells subjected to osmotic downshift are presented in Figure 6.6.

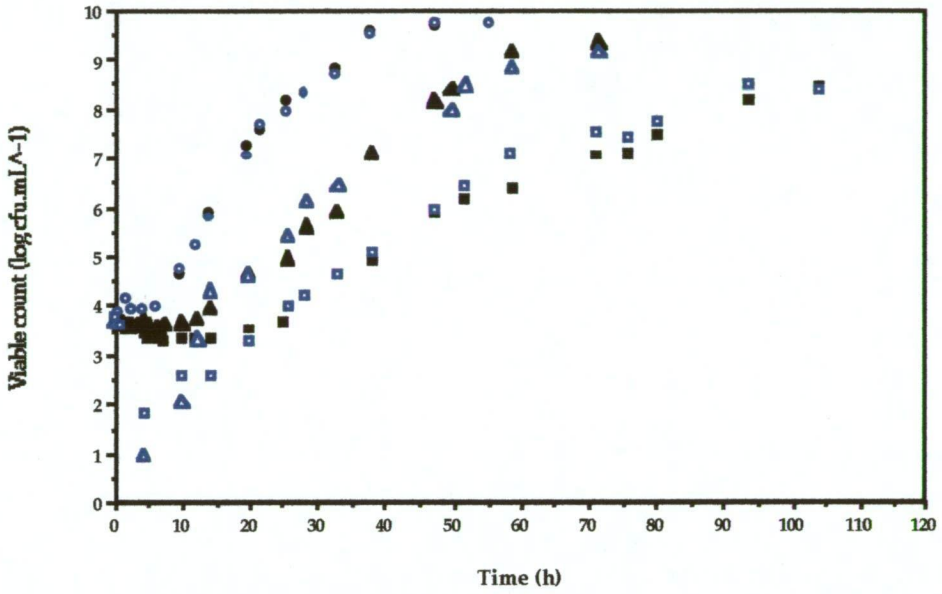

Figure 6.6: Effect of osmotic downshift from a_w 0.995 on growth of stationary (black) and exponential phase (blue) *S.* Typhimurium at a_w 0.976 (●/○), 0.964 (▲/△) and 0.956 (■/□).

Inoculum history appeared to have little effect on the shape of the growth curve, and hence growth rate, through the exponential phase of growth at each a_w tested. In comparison to analogous stationary phase cultures, growth curves from exponential phase cells showed some injury at a_w 0.964 and 0.956, but this was resolved within the lag phase. Lag phase duration appeared similar for both treatments at each a_w. MPD decreased as the a_w decreased.

The RLT response of exponential and stationary phase cells monitored by viable count increased as the magnitude of the osmotic shift increased (Table 6.1). Exponential phase cells had a slightly higher RLTs than stationary phase cells at lower a_w; a difference of 0.3 and 0.9 respectively.

Table 6.1: RLT of exponential and stationary phase *S.* Typhimurium determined by viable count

Inoculum	RLT		
	a_w 0.976	a_w 0.964	a_w 0956
Stationary	3.9	5.7	6.5
Exponential	3.9	6.0	7.4

6.3.2 Diauxic growth

6.3.2.1 Effect of addition of glucose on RLT

The RLT for *S.* Typhimurium in basal BHI was similar to that presented in Figure 5.10, i.e. RLT increased as the magnitude of the shift increased to a maximum of ~8 at a_w 0.965 (Fig. 6.7). The expected RLT 'downturn' in basal BHI was not observed as none of the media was of sufficiently low a_w. The RLT response of *S.* Typhimurium in BHI with 0.6% added glucose increased with the magnitude of the osmotic shift, to a maximum value of 10 at a_w 0.960. The RLT response of *S.* Typhimurium in BHI with 2% added glucose increased with the magnitude of the osmotic shift to a maximum of ~17 at a_w 0.956. The data for the second experiment in BHI with 2% added glucose (Fig. 6.8) are also plotted with data from Figure 6.7. Raw data are presented in A. 9.4.23.

The RLT response confirms that described above. Data at a lower a_w were available, and this increased the maximum RLT value observed to 18 at a_w 0.954.

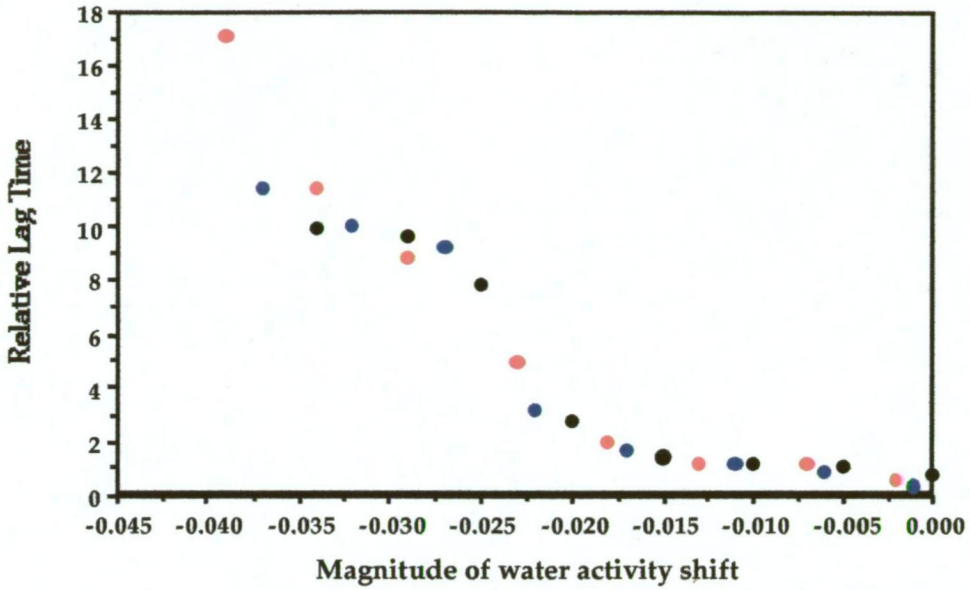

Figure 6.7: Effect of a_w downshift on the relative lag time response of exponential phase *S.* Typhimurium in BHI, (●), BHI with 0.6% (●) and 2% (●) added glucose.

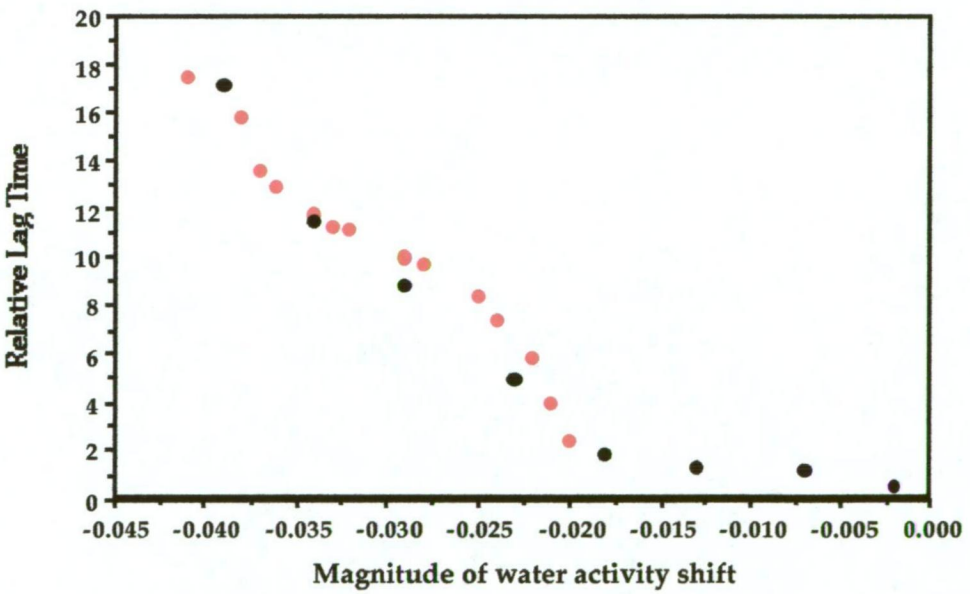

Figure 6.8: Effect of a_w downshift on the relative lag time response of exponential phase *S.* Typhimurium below a_w 0.980 in BHI with 2% added glucose (●). RLT data from Figure 6.7 in BHI with 2% glucose are also plotted (●).

The growth rate and lag rate of *S.* Typhimurium in BHI with 2% added glucose are presented in Figure 6.9. Growth rate declines in a biphasic pattern with decreasing a_w. This is similar to that observed for *K. oxytoca* in BHI at a_w's below 0.970 (Fig. 5.4). For lag rate the response is more pronounced.

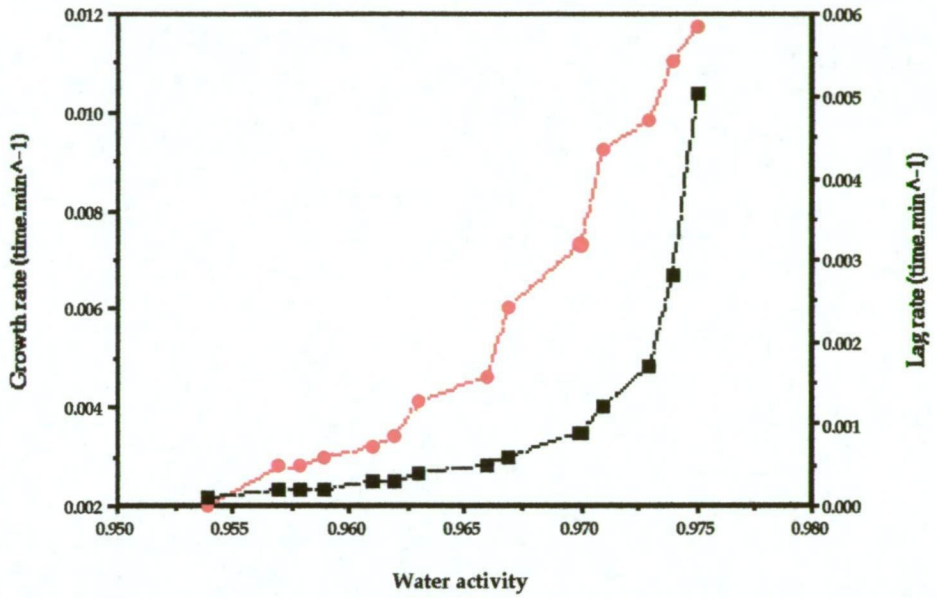

Figure 6.9: Effect of a_w downshift on the growth rate (●) and lag rate (■) response of exponential phase *S.* Typhimurium in BHI with 2% added glucose.

The growth rate data for all *S.* Typhimurium and a_w downshifts in basal BHI, BHI with 0.6% added glucose, and BHI with 2% added glucose (for which there are two data sets) are presented in Figure 6.10. To exemplify the differences in growth rate at low a_w, only part of the growth rate curve is plotted, i.e. for shifts below ~a_w 0.975 (corresponding to a shift of > -0.020 a_w units).

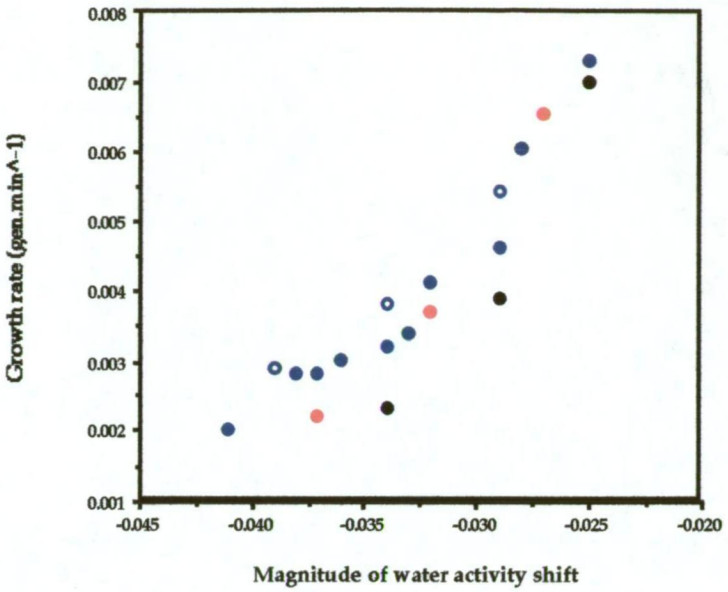

Figure 6.10: Effect of a_w downshift below ~a_w 0.975 on the growth rate of exponential *S.* Typhimurium in BHI(●), BHI with 0.6% added glucose (●) and BHI with 2% added glucose (●= data from 1st experiment and ○= data from 2nd experiment).

Although there are relatively few data for the BHI and BHI with 0.6% added glucose, a trend in growth rate and media composition is noticeable. Inclusion of glucose in the basal laboratory media increases apparent growth rate at low a_w. The effect is more pronounced for 2% added glucose in comparison to 0.6% added glucose. Differences in the growth rate between each media type could not be measured as there are no a_w values for which all 3 data types are represented. However, from the general trends observed, after a a_w shift of approximately –0.025 a_w units there are only small differences in the growth rates for the different media types and are all <10%. At a lower a_w corresponding to a shift of ~-0.035 a_w units the growth rate is ~20 to 50% faster in BHI with 2% added glucose and ~15 to 30% faster in BHI with 0.6% glucose added in comparison to the growth rate in basal BHI.

6.3.2.2 RLT of exponential *K. oxytoca* in BHI with 2% added glucose.

The addition of 2% glucose to basal BHI did not alleviate the 'downturn' in RLT of *K. oxytoca* (Fig. 6.11). As described previously, RLT increased as the

magnitude of the shift increased. A maximum of 6.4 was reached at a_w 0.955 when glucose was included in the outgrowth medium. This compares to a maximum RLT of 6.3 at a_w 0.958 in basal media (Fig. 5.8). Raw data are presented in A. 9.4.24.

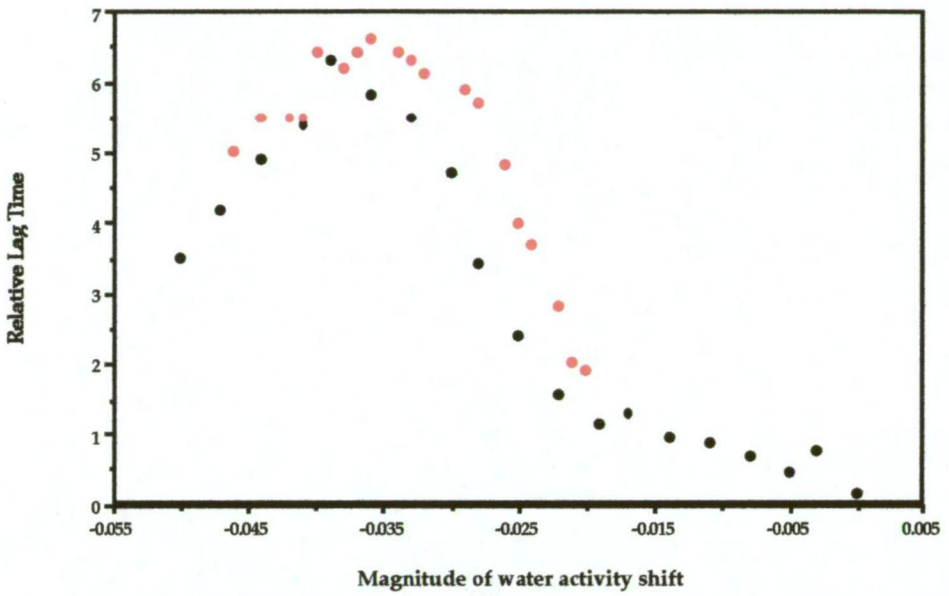

Figure 6.11: Effect of a_w downshift on the relative lag time response of exponential phase *K. oxytoca* below a_w 0.980 in BHI with 2% added glucose (●). RLT data from Figure 5.8 in basal BHI are also plotted (●).

6.3.3 RLT reproducibility and variability

6.3.3.1 Reproducibility

RLT data for a_w downshifts from two separate experiments for late exponential phase *E. coli* SB1 are presented in Figure 6.12. In general RLT values are similar. Small differences (~1 RLT) occur in the medium to high a_w range where RLTs are <2. Raw data are presented in A. 9.4.9 and A. 9.4.25.

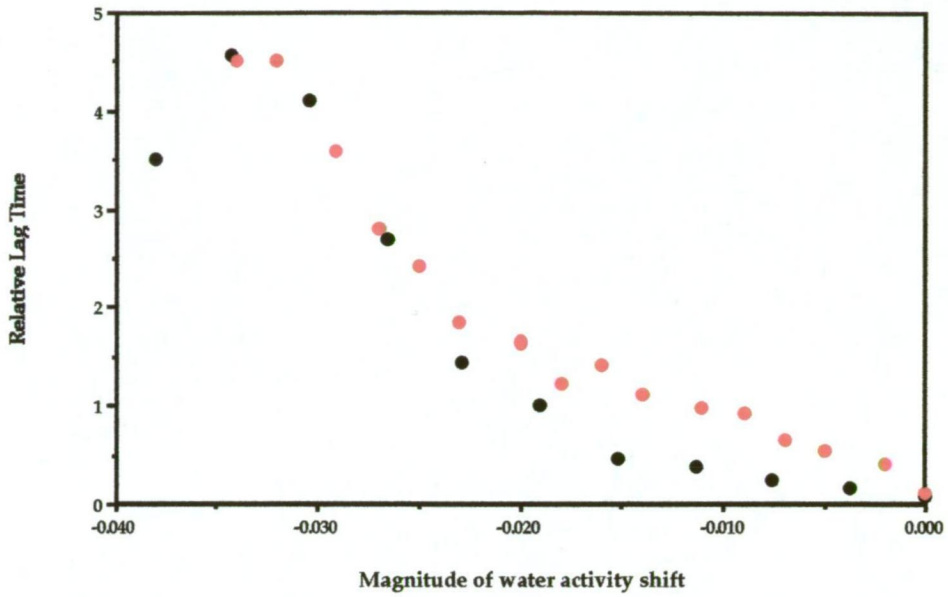

Figure 6.12: Effect of a_w downshift on the RLT of *E. coli* SB1 where ● =data from Figure 5.5 and ● =new data

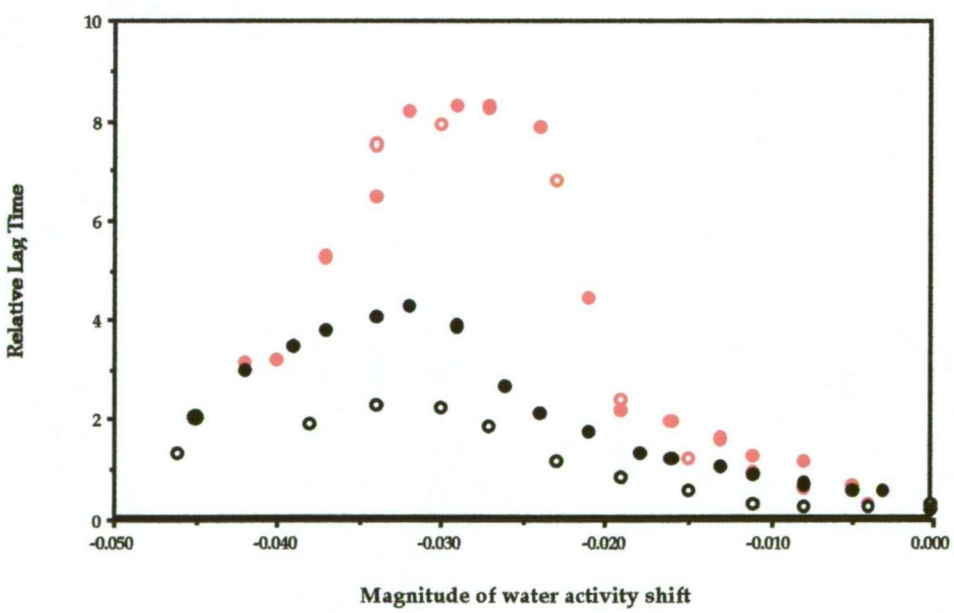

Figure 6.13: Effect of a_w downshift on the RLT of *S.* Typhimurium where open symbols are for data from Figure 6.1 and closed symbols are for data from Figure 6.2. Stationary phase data are black and exponential phase data are red

RLT data for a_w downshifts for exponential and stationary phase *S.* Typhimurium are presented in Figure 6.13. For exponential phase inocula, the RLT response from separate experiments correlates well. As for *E. coli* SB1, small differences in RLT (~1) occur in the mid to high a_w range where RLTs are <2. For stationary phase inocula the RLT response follows the same pattern, however, the RLT values from the second data set (from Fig 6.2) are approximately 1 to 1.5 RLT units higher.

6.3.3.2 Simulations

Growth curves based on the pattern presented in Figure 6.3 were generated with different slopes. The generation and lag time estimates for each curve are presented in Table 6.2.

Table 6.2: Effect of variations in the slope of the exponential phase of an idealised growth curve on kinetic growth estimates

% variation in slope from "true"	GT	LT	RLT	% difference from "true" RLT
0	0.301	1.000	3.3	0
5	0.317	0.947	3.0	-10.1
10	0.334	0.889	2.7	-20.1
15	0.354	0.824	2.3	-30.2
20	0.376	0.750	2.0	-40.3
25	0.401	0.667	1.7	-50.3
30	0.430	0.571	1.3	-60.4
35	0.463	0.462	1.0	-70.5
40	0.502	0.333	0.7	-80.5

Reducing the slope by 5% intervals increased generation times and decreased lag times. Consequently RLT was progressively decreased. The decrease in RLT was regular, ~10% for each 5% change in slope.

The data for exponential *S.* Typhimurium in basal BHI (Fig 6.5) are plotted with that for exponential *S.* Typhimurium in BHI with 2% added glucose (Fig. 6.11) in Figure 6.14. RLT points at similar a_w in the 'downturn' area of the plot are marked with closed symbols.

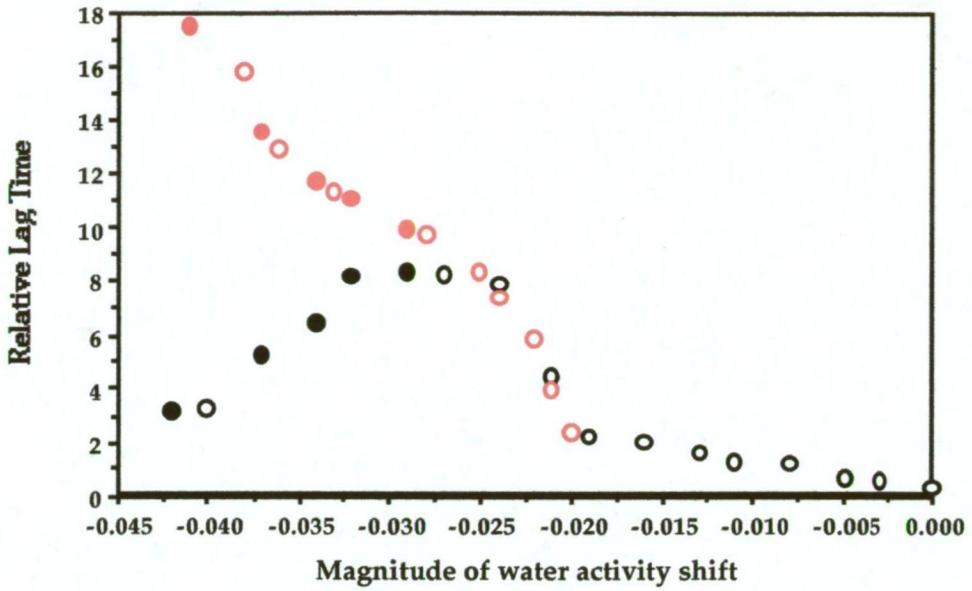

Figure 6.14: Effect of a_w downshift on the RLT of exponential *S.* Typhimurium in BHI (○), data from Fig. 6.5, and BHI with 2% added glucose (○), data from Fig. 6.8. Closed symbols indicate points for RLT comparison.

The RLT values of the two treatments differ by values of approximately 20, 40, 50, 60 and 85% as the a_w is decreased.

6.4 DISCUSSION

6.4.1 Physiological history effects on RLT

In the case of *S.* Typhimurium and a_w downshifts, the physiological history of the inoculum had an effect on RLT. This effect was more notable for turbidimetric estimates of RLTs than viable count RLTs. RLT values for stationary phase cells were much smaller than for exponential cells, i.e. more metabolically active cells had longer lag phases. This is in contrast to Baranyi and Roberts (1994) who hypothesised that exponentially growing cells have a shorter lag phase than analogous stationary phase cells if an environment shift is undertaken. However, their work is theoretical and does not provide details on the type and extent of environmental shift required to produce this response. In the

experiments conducted in this chapter, the post- transfer environment is progressively harsher and shifts often large.

The disparity in the RLT response for exponential and stationary phase cells may arise for a number of reasons. Firstly, stationary phase cells are generally considered more stress resistant than their exponentially growing counterparts. It is possible that these cells are able to resolve lag in an osmotically stressful environment more quickly than exponential phase cells. Secondly, in the case of turbidimetric RLT estimates, differences in population size between exponential and stationary phase inocula may result in smaller RLTs for stationary phase cells as larger populations are considered to have smaller lag phases (Baranyi and Roberts, 1994).

In stationary phase general defence systems are developed which prepare cells to survive very different environmental stresses, without the need for cultures to have had prior exposure to them (Pichereau *et al.*, 2000). Many of these genes are under the control of alternative stress and stationary phase sigma factors, σ^S, in gram-negative and σ^B in gram-positive species. The response to hyperosmolarity shares a number of similarities with the stationary phase response induced by starvation, as (i) the global stationary phase regulon σ^S and σ^B respond to hyperosmolarity and (ii) overlaps between osmotic stress and starvation induced multitolerances often occur in bacteria (Jenkins *et al.*, 1990; Hengge-Aronis, 1996). Some systems directly involved in protection include *ots*BA involved in the synthesis of trehalose and *pro*U and *pro*P encoding two general osmoprotectant uptake systems (Pichereau *et al.*, 2000).

Stationary phase *E. coli* subjected to lethal osmotic challenges have enhanced osmotic resistance in comparison to mid-logarithmic phase cells, or cells preadapted to osmotic stress (Jenkins *et al.*, 1990). This enhanced resistance due to the presence of stress proteins may also allow stationary phase cells to exploit new growth permissive environments more rapidly than exponential phase cells. The general stress response involves some osmoregulatory mechanisms. As these are already in place stationary

phase cells may have comparatively less *work* to do to resolve lag under osmotically stressful conditions than exponential phase cells. These shorter lags would in turn lead to smaller RLT values for stationary phase cells. The data for the 'controlled mixes' of inocula support this hypothesis (Fig. 6.4). Predominantly stationary phase inocula gave a similar response to stationary phase cells. Although the exponential phase component of the inoculum would contribute to growth in the new environment, the stationary phase cells are expected to resolve their lag more quickly and, in turn dominate the growing population. The predominantly exponential phase inocula had an intermediate RLT response. It is likely the stationary phase cells resolve lag earlier in this situation and dominate the population, but due to smaller numbers than the purely stationary phase inoculum the lag phase of the entire population is longer. The late exponential phase inocula used for experiments conducted in Chapter 5 is of an unknown mix of cells. Comparing the RLT response for this inoculum (Fig. 6.5) to the 90% exponential phase inoculum (Fig. 6.4) suggests that there is >90% exponential cells as the RLT response is slightly higher.

Morphological changes occur in stationary phase. The familiar rod growing shape of *E. coli* is lost in stationary phase because cells become much smaller and almost spherical as the result of several cell divisions without an increase in cell mass (Ingraham *et al.*, 1983). Thus, although the a_w outgrowth media is inoculated in such a way that the starting %transmittance is the same, there are more stationary phase cells per mL than exponential phase cells. The difference in population size for the exponential and stationary phase inocula was measured as 1.1 log cfu.mL^{-1}. A one log difference in numbers may result in the stationary phase cells having a shorter lag phase. With regard to population effects on lag phase duration, there are conflicting reports in the literature. Some proponents claim that inoculum size has no effect on lag phase duration (Mackey and Kerridge, 1988; Buchanan and Cygnarowicz, 1990; Palumbo *et al.*, 1991; Augustin *et al.*, 1999). In contrast, Ross (1993) and Baranyi (1998) suggest that population size will have an effect due to the distribution of cells within a population. Many of the reports in the literature are for optical

density data, and as yet there seems to be no systematic and rigorous investigation of the effect of inoculum size by viable count.

The viable count experiment for exponential and stationary phase cells at 3 different a_w's does not provide as clear a RLT pattern as the turbidimetric data. The outgrowth conditions at low a_w may be quite injurious, and cells enumerated by viable count on an enhanced resuscitation medium may have a greater opportunity to recover than those cells which remain in the broth system. Alternatively, the relatively low RLTs determined by viable count may be an artifact due to diauxic growth. Closer examination of the plots in Figure 6.6 reveals progressive changes, i.e. as a_w is lowered, in the exponential portion of the growth curves is indicative of diauxie. The disparity in viable count and turbidimetric data is not a unique observation, and potential differences in estimations of kinetic data have been discussed previously (S. 3.1.3). Hudson and Mott (1994) found lag times from optical density data for *Pseudomonas fragi* were shorter than viable count data. Wu *et al.* (2000) observed that mean individual cell lag times of *L. monocytogenes* obtained by the Bioscreen method were longer than those found microscopically. The Bioscreen is a turbidimetric instrument and thus an indirect method for growth curve generation time.

It is possible that the potential effect of differences in population size, discussed above, may be exacerbated if the number of exponential phase cells is decreased due to injury or cell death. Dead or injured cells contribute to optical density measurements and mask the growth of uninjured cells, hence lag phases appear artificially long. The viable count data certainly demonstrate that injury is occurring for exponential phase cells inoculated into outgrowth media at low a_w
(Fig. 6.6). It is noteworthy that injury is observed even on a non-selective and enhanced resuscitation medium designed to give maximal recovery of injured cells.

The L-tube system employed in the turbidimetric experiments is also a highly aerated system which may expose cells to oxidative stress. Dodd *et al.* (1997) suggested an additional element for the differential sensitivity

between exponential and stationary phase cells is that, for exponentially growing cells, some of the lethal effects associated with inimical processes result from a process of 'self destruction' by the cells; a suicide response. This is caused by a near instantaneous oxidative burst which occurs when cells are growth-arrested following an inimical treatment. The imposed stress disturbs cellular homeostasis which leads to growth inhibition but does not affect metabolic rate, i.e. cell division is decoupled from metabolism. This results in overactive and perhaps futile metabolism that produces intracellular free radicals which are lethal to unadapted cells. Protection against self-destruction can be provided by reducing the oxygen tension, or by adaptive responses associated with the stationary phase which protect the cell against DNA damage, free-radical damage and protein denaturation. It is possible that oxidative stress may reduce the number of cells in the exponential phase population, thus inducing longer lag phases. This hypothesis requires further examination.

From Figure 6.4, it appears that stationary phase inocula subjected to a_w downshifts have an RLT response which is up to 4 RLT units less than exponential phase cells. Knowledge of the physiological state of the inoculum is important in defining meaningful RLTs, and this may affect the design and interpretation of future experiments. It should be noted that in previous experiments describing the effect of abrupt environmental shifts on lag phase duration (Chapter 5) inocula were considered to be 'late exponential' or 'mixed phase'. While inoculum preparation was standardised for experiments involving the same strains, it is likely that for different strains the 'late exponential phase' cultures contained different proportions of exponential and stationary phase cells due to individual growth characteristics at the incubation temperature of 25 (±0.1)°C. In the experiments described above, the late exponential phase culture of *S.* Typhimurium gave an intermediate response between purely exponential and stationary phase cultures (Fig 6.5). In that instance the initial experiments provide an 'average' RLT response to osmotic shifts which may be useful for inclusion in predictive models or risk assessments. However, osmotic shift experiments should be repeated for other strains to determine if the late exponential data is an average

response, or whether curves are closer to either purely exponential or stationary phase results.

6.4.2 Diauxic growth

Supplementation of basal BHI medium with glucose alleviated diauxic growth of exponential phase *S.* Typhimurium at low a_w. Growth curves were more classically sigmoidal in shape and more realistic lag and generation time estimates were generated by linear regression.

At the low a_w values, below ~a_w 0.970, differences were observed in growth rate of exponential *S.* Typhimurium in the various types of media. Growth in BHI supplemented with glucose was faster than in basal BHI. This difference in growth rate was estimated to be between 40 and 60%, dependent on the amount of added glucose. More rapid growth at low a_w is an important observation in the context of RLTs, as it will increase the RLT value. At low a_w, variation in growth rate estimates was evident between two separate experiments where 2% glucose was included in the outgrowth medium.

The 'downturn' in RLT for a_w downshifts was eliminated by inclusion of 2% glucose in the laboratory medium. The absence of a 'downturn' in RLT increased the observed maximum RLT to ~18. Comparable experiments in basal media without added glucose yielded a maximum RLT of 8 (Figures 6.4 and 6.5). This changes the pattern of RLT distributions presented in Figure 5.35, lengthening the right hand tail. Large shifts in a_w to conditions close to the lower limits for growth produce large RLTs.

Unlike *S.* Typhimurium, inclusion of additional glucose in the outgrowth medium at low a_w did not resolve the 'downturn' in RLT for *K. oxytoca.* In fact, RLT plots were similar to that for unsupplemented BHI (Fig. 6.9). This result was surprising as *K. oxytoca* has no special growth factor requirements and can utilise glucose as a sole carbon source (Ørskov, 1984). It is possible that a switch from oxidative to fermentative metabolism occurred for *K. oxytoca* resulting in acid end products. A square-root type model for *K. oxytoca* developed in Chapter 2 estimated pH_{min} to be 4.7. If the pH of the outgrowth media did change due to

fermentative metabolism and approached the lower pH limits for growth, a diauxic growth rate pattern would occur and affect RLT estimates. This requires further investigation.

It was difficult to determine whether there was a minimum level of additional glucose required to eliminate diauxie for *S.* Typhimurium as there were insufficient data collected at the lowest a_ws for media containing 0.6% added glucose (Fig. 6.7). However, BHI contains 0.3% glucose and the results suggest levels higher than this are required to eliminate diauxic growth under the most extreme conditions.

It was reported in S. 6.3.1 that physiological history of the inoculum affected RLTs at low a_w for turbidimetric experiments, but the result was less clear for viable count data. Diauxic growth is evident in the viable count growth curves for exponential and stationary phase outgrowth at low a_w's (Fig. 6.6). This may account for the similarity in the RLT value for a shift to that a_w and the shift to a_w 0.964 (*see* Table 6.1). The generation and lag time estimates used in the calculation of RLT may be unrealistic and falsely low, and larger differences might be observed if additional glucose is included in the outgrowth medium. This requires further investigation.

6.4.3 Reproducibility and variability

RLTs were reproducible for two of the three data sets examined. For all data there were small differences in RLT, ~1, for small a_w downshifts, i.e. in the range characterised by relatively fast growth rates and small lag times. It was suggested in S. 5.4.1.1. that there may be a limit of resolution for RLT determinations in the range 0 to ~2 where the effects of true, physiologically-based, responses are difficult to distinguish from 'noise' due to the lack of sensitivity of turbidimetric measurement and the ratio itself. For the third data set, stationary phase *S.* Typhimurium, RLTs were up to twice as large. However, for one data set RLTs were ≤2, therefore there is the possibility that the disparity in the RLT plots could be due to lack of resolution at low RLT values. Interestingly the RLT plots were more reproducible for experiments where late exponential and purely exponential phase inoculum were used. To clarify if reproducibility is

affected by the physiological history of the inoculum further experiments are required. Confirmation of reproducibility by viable count would also be useful.

Altering the slope of an idealised growth curve provided information on how much of a change in the exponential portion of bacterial growth would affect RLT estimates. A small change in slope, 5%, was sufficient to alter the RLT estimate by ~10%. A larger change in slope, 40%, would alter the RLT estimate by ~80%. The difference in RLT estimates for exponential *S.* Typhimurium in BHI and BHI with 2% added glucose was estimated at 20-65% (Fig. 6.14). Taking the most extreme values, i.e. RLTs of ~3 and ~18, this 84% difference in RLT corresponds to a change in slope of 40-45%. This correlates well with the observed differences in growth rate estimated to be up to 50% at the lowest a_w (Fig. 6.10).

It is evident that a relatively minor miscalculation in the slope of the exponential phase can have a large affect on RLT. Minor miscalculations due to experimental error are likely. However, relatively large and systematic differences in slope are required (*see* Table 6.2) to account for the 'downturn' in RLT observed for gram- negative organisms at low a_w. Diauxic growth was identified as a contributing factor, however the methodology used to generate RLT data also contributes.

Much of the data presented in this thesis was derived turbidimetrically. Turbidimetry has a low sensitivity limit and is unable to detect populations less than about 10^7 cfu.mL^{-1}. Consequently only a narrow region of the growth curve is measured and may not be representative of the population at maximum exponential growth (Dalgaard *et al.*, 1994). Thus, as the maximum population density decreases progressively as a_w is decreased (*see* Fig 6.6), it is possible that growth rates from subsequent curves are systematically underestimated. It was observed that changes in the exponential portion of viable count growth curves also occur at low a_w. Data for outgrowth of exponential and stationary phase *S.* Typhimurium at a_w 0.956 (Fig. 6.6) are plotted in Figure. 6.15.

Figures 6.6 and 6.15 show that the alteration in the shape of the growth curve at low a_w begins at ~$1x10^6$ cfu.mL^{-1}. In the turbidimetric experiments described in this chapter, starting numbers in L-tubes were ~$5x10^6$ cfu.mL^{-1} for exponential phase inocula and ~$2x10^7$ cfu.mL^{-1} for stationary phase inocula, and are marked in Figure 6.15. Thus, when growth curves are measured turbidimetrically at low a_w the maximum exponential growth rate of the organism is not observed, even prior to diauxic growth or other phenomena altering the shape of the growth curve. As a_w levels become progressively more restrictive to growth the effect is more pronounced, i.e. the exponential growth rate is effectively being measured at later and later stages in the growth curve. This leads to systematically increasing underestimations of the maximum specific growth rate. An increase in the generation time, i.e. decreasing growth rate, and the related decrease in lag time estimate decreases RLT, causing the unexpectedly small RLT estimates observed as a 'downturn' at low a_w.

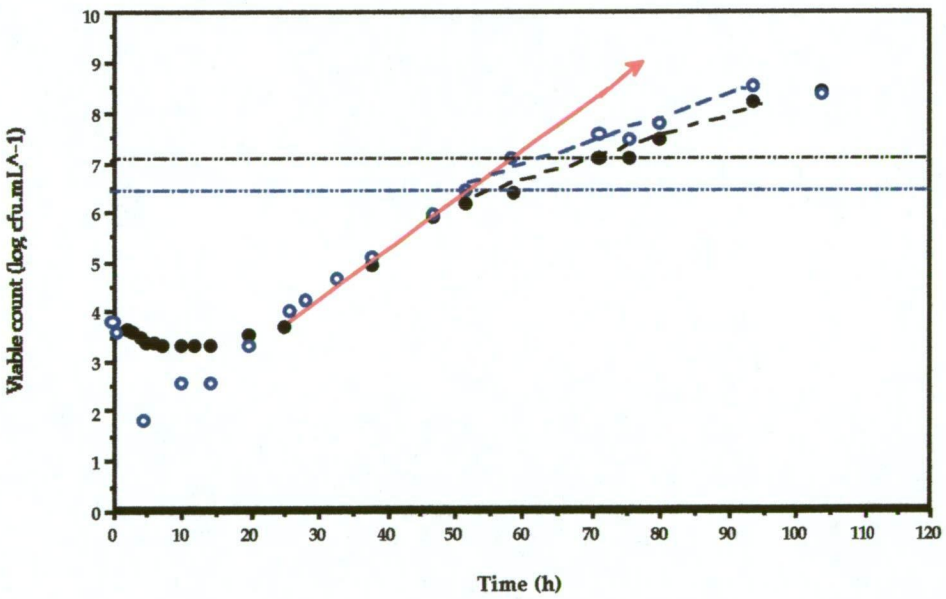

Figure 6.15: Growth of exponential (○) and stationary phase (●) *S.* Typhimurium in BHI (○) determined by viable count, data from Fig. 6.6. To illustrate diauxic growth, the potential shape of the exponential portion of the curve in the presence of the preferred substrate is marked (---) and growth on the secondary substrate marked with dotted lines. Inoculum levels for turbidimetric experiments are marked with (---) for exponential inocula and (---) for stationary phase inocula.

6.4.4 Summary

- The physiological history of a bacterial population affects the RLT response of *S.* Typhimurium for a_w downshifts. More metabolically active cells, i.e. exponential phase, have a larger RLT response than cells whose activity is low, i.e. stationary phase. It is hypothesised that stationary phase cells are able to exploit new growth environments at low a_w more effectively than exponential phase cells, resulting in lower RLTs.

- The effect of physiological history on RLT is clearest for turbidimetrically generated data in comparison to viable count. However, injury and 'cell suicide' effects may result in population differences which make comparisons difficult. These hypotheses require further investigation.

- Careful interpretation of RLT responses under very stressful conditions is required due to potential changes in growth curve shape. Small changes in the exponential portion of a bacterial growth curve translate to large changes in RLT.

- For *S.* Typhimurium, the 'downturn' in RLT was relieved by the addition of glucose to outgrowth media at low a_w. For *K. oxytoca,* addition of glucose did not affect the RLT 'downturn'. The latter result was unexpected and remains unexplained.

- RLTs are reproducible.

7. SUMMARY AND CONCLUSIONS

Two areas of predictive modelling relevant to the microbial safety of meat were examined in this thesis. Firstly, a new square-root type model for *E. coli* growth was proposed and its performance assessed and a predictive square-root type model for *K. oxytoca* was developed for use in carcass chilling trials. Secondly, the effects of abrupt temperature, pH and osmotic shifts on the growth of *E. coli* and other foodborne bacteria were examined. In particular, effects on lag phase duration were investigated and the relative lag time concept was used to characterise these effects.

The predictive model for growth of *E. coli* presented in this thesis was shown to perform very well for meat-specific data, and to outperform other models for *E. coli*. The lactic acid terms in the model were hypothesised to account for this good performance. This emphasises the value in using in industry predictive models which include parameters specifically relevant to the product.

The results from the investigation of a_w mediated sub-lethal injury (Fig. 3.33 and 3.34) may have significance in relation to the evaluation of carcass chilling regimes or carcass decontamination procedures. An awareness of such sub-lethal injury is essential when conducting viable counts for hygiene monitoring or assessment of decontamination procedures, and for interpretation of those results. Reports in the literature on some carcass decontamination processes have shown that effects are relatively short lived (Dickson and Anderson, 1992; Dickson *et al.*, 1994). Those workers report that, in some cases, the microbial loads on a carcass that has undergone a decontamination step return to those of the untreated carcass after more prolonged storage. Similarly, in this study osmotically injured cells have been shown to repair injury.

The results of the temperature, pH and osmotic shift experiments indicate that if the change in environment is severe enough, lag phases are induced. As temperature and a_w are known to exert an effect at the carcass surface, development of new chilling strategies which decrease temperatures and dry the carcass surface more rapidly than current techniques may reduce microbial proliferation on carcasses. Such

strategies would also have to be compatible with product quality and technological considerations.

The similarity in the observations from the temperature, pH and osmotic shift experiments and other results (Ross 1999), provides confidence that RLTs in the range 4 to 6 are commonly observed. It would appear that there is now a substantial body of information to *justify* the inclusion of lag times in calculations of the effects of different meat processing and handling procedures. This is significant for predictive microbiology in general, and specifically for the meat industry where it may have implications for carcass chilling and other meat processing and handling operations. For example, from a practical perspective, a lag time of 3 generation time equivalents (i.e. RLT) reduces the expected growth *without lag* by 0.9 log CFU, i.e. almost a factor of ten. Similarly, four or six generations times of lag equate to a reduction in the expected growth of 1.2 and 1.8 log CFU respectively. Such differences due to lag in terms of the amount of growth expected is important in terms of food safety regulations limiting the extent of possible growth during a particular process to a specific number of log cycles.

As predictive modelling and risk assessments have traditionally used 'worst-case' scenarios for lag time estimations, the inclusion of low RLTs is likely. However, the data presented here demonstrates that under some conditions for a single strain, large RLT values are generated. This emphasises that a knowledge of the process, pathogen and product under investigation is extremely important when making decisions about inclusion of RLT in predictions. Another consideration that arises is the likelihood of preferential utilisation of substrates occurring in foods and the effect this will have on RLT estimates.

The results presented in this thesis contribute towards realising the potential of predictive modelling in the food industry and characterising bacterial lag times to improve the microbiological quality and safety of foods. Significant outcomes from this study include:

- Model performance can be assessed objectively by comparison to large sets of independent data using the bias and accuracy factors.

- A new model for *E. coli* growth rate was developed and evaluated.

- The lag phase can be interpreted in terms of the *amount* of work to be done and the *rate* at which this work is done.

- Although the relationship between growth environment and lag phase duration is variable, this variability can be reduced by applying the relative lag time concept.

- Relative lag times are reproducible if the prior history of the cell is known.

- There may be a lower limit of resolution for RLT if turbidimetric techniques are used.

- Alterations in the shape of bacterial growth curves can affect RLT estimates. Normal experimental error and diauxic growth were identified as two potential sources of error.

- Variations in kinetic growth data between turbidimetric and viable count techniques are important considerations in model performance evaluations and interpretation of RLT responses.

- The results from the lag time experiments offer the prospect of inducing bacterial lags to improve shelf life and safety of foods by manipulating the rate and extent of change of environmental conditions.

- The presence of sub-lethally injured cells must be considered when monitoring process control in abattoirs and for hygiene assessments.

- A critical a_w value at which 'cell yield' begins to decline is hypothesised to denote the lower limit of a normal physiological range for a_w.

- The concept of predictive microbiology as a useful tool to the meat industry is supported by the preliminary results from a carcass chilling verification study.

8. REFERENCES

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9. APPENDICES

9.1 STRAIN DETAILS, MAINTENANCE AND RECOVERY

9.1.1 Bacterial strains

E. coli M23	Non-pathogenic strain from cryogenic culture, courtesy of Dr K Sanderson, University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001.
E. coli SR-M23	Streptomycin resistant mutant of *E. coli* M23 isolated by Ms L Mellefont, University of Tasmania.
E. coli R31	Verotoxigenic clinical strain from slope culture courtesy of Dr M Salter, University of Tasmania.
E. coli SB1	Non-pathogenic clinical isolate from Ms S. Bettiol, Department of Pathology, University of Tasmania.
K. oxytoca (NRRL B-199)	Non-pathogenic strain from slope culture courtesy of Mr I Eustace, Australian Meat Technology, Brisbane
S. Typhimurium (M48)	Non-clinical strain from cryogenic culture, courtesy of Dr K Sanderson, University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001.
S. aureus (ATCC 25923)	Type strain from cryogenic culture courtesy of "Aquahealth" (Microbiological Consultancy), University of Tasmania.
L. monocytogenes (L5/22)	A wild type cold smoked salmon isolate from slope culture, courtesy of Dr S. Tienungoon, University of Tasmania.

9.1.2 Cryogenic storage

Bacterial cultures for all experiments were stored at -70°C. Cultures were maintained in duplicate, with one set held for subculture purposes only.

Plastic 3mm beads were cleaned with a tap water and detergent wash followed by a rinse in dilute HCl to neutralise alkalinity. The beads were then washed several times in tap water, followed with a final distilled water rinse and air drying. Approximately 20-30 beads were placed in 5mL screw-cap glass vials and sterilised by autoclaving at 121°C at 15 psi for 15 minutes.

Each bacterial strain was grown for 24 hours on Columbia Horse Blood Agar (CHBA) at 35°C. The plate was harvested by pipetting 1mL of sterile Nutrient Broth with 15% glycerol added (NB-Gly) onto the surface of the plate then emulsifying the growth with a wire loop. The bacterial suspension was aseptically pipetted into two vials containing sterile prepared beads. The suspension was aspirated several times to ensure displacement of air in the beads. Excess suspension was removed then vials were placed on their sides and the beads distributed by gentle tapping in order to facilitate easy removal. Vials were placed at -20°C for 24 hours before transfer to -70°C for storage up to 7 years.

9.1.3 Recovery from cryogenic storage

Cultures on beads were recovered by aseptically removing one bead from the vial while on ice in order to prevent excessive heating of the culture. The bead was rubbed gently over the surface of a CHBA plate. The inoculated plate was incubated at 35°C for 24 hours. The culture was checked visually for purity (colony morphology only) then plated onto an appropriate medium to test for typical reactions.

9.2 EQUIPMENT

Multi-well Plates	Falcon® 3043-12 well flat bottom tissue culture plate (Becton Dickinson and Co., 2 Bridgewater Lane, Lincoln Park, New Jersey 07035, USA).
O_2 permeable film	Parafilm "M" laboratory film-4 inch (America National Can™, Chicago, IL 60631, USA).
pH Meter and electrode	Orion pH meter 250A (Orion Research Inc. Boston, MA 02129, USA), and flat tip probe.

The instrument was calibrated on each occasion before use by reference to buffers at pH 4 and pH 7.

Pipettors	Fixed and variable pipettors were used: •100μL and 1000μL-variable (Gilson Medical Electronics, B.P. 45, F95469 Villiers-le-Bel, France). •0.01-10.00mL-electronic digital pipette (Rainin Instrument Co., Inc., 5400 Hollis St, Emeryville, CA 94608-2508).

The dispensed volume of all pipettors was checked by weighing of water at room temperature prior to use. This volume was typically found to be within ±1% of the nominal volume.

Spectrophotometer -analogue	Spectronic 20+, Milton Roy Co., USA
Spectrophotometer -digital	Spectronic 20D, Milton Roy Co., USA
Spiral Plater-Autoplate 4000	Spiral Biotech Inc., 7830 Old Georgetown Rd, Bethesda, MD 20814 USA
Stomacher Stomacher Bags-17.7x30.0cm	Colworth 400, A.J. Seward, London, UK. Bio-Service Pty . Ltd., P.O. Box 180, Huntingdale, Vic, 3166, Aus.
TGI-1	Advantec TN-2148, Advantec MFS, Inc., 6691 Owens Drive, Pleasanton, CA 94588, USA.
TGI-2	Terratec Asia-Pacific Pty., Ltd. Lot 7, Patriarch Dr, Kingston, Tasmania, 7054, Australia.
TGI-3	Advantec TN.3, Advantec MFS, Inc., 6691 Owens Drive, Pleasanton, CA 94588, USA.
Thermometer	Fluke® 51K/J (John Fluke Mfg. Co. Inc., 1150 Euclid Avenue, Palatine, IL 60067, USA) electronic thermometer with Iron-Constantan thermocouple bead probe.
Water Activity Meter:	Aqualab CX-2 (Decagon Devices, Inc., PO Box 835, Pullman, Washington 99163, USA).

The instrument was calibrated on each occasion before use by reference to distilled water and to a saturated salt (NaCl, a_w 0.953) standard.

Waterbath	Ratek SWB20D shaking waterbaths, Ratek Instruments Pty. Ltd., Unit 1/3 Wadhurst Drive, Boronia, VIC, Australia, 3155.

9.3 MEDIA

9.3.1 Media preparation

Basal media was prepared as per manufacturers instructions unless otherwise stated. Addition of supplements was as per manufacturers instructions or as described in this section. Sterilisation was by autoclaving at 121°C at 15 psi for 15 minutes (unless otherwise specified) or in the case

of non-sterile, heat sensitive supplements by filter sterilisation. Where necessary pH was modified by the addition of 0.1M NaOH or 0.1M HCl unless otherwise stated. pH was measured post-autoclaving and adjusted aseptically by the addition of sterile HCl or NaOH if required. For NaCl modified media, a_w was determined from triplicate readings. All water used in the preparation of media was prepared by glass distillation of tap water.

Quality control was exercised on each batch of media prepared by:

- measurement of media pH after sterilisation
- incubation of an uninoculated plate at 25°C for 40 to 48 hours as a sterility check
- growth of target organism at optimal temperature

Media failing these criteria were discarded.

Media were stored at 4°C for up to 4 weeks. Media containing antibiotics was stored in the dark at 4°C for up to 2 weeks.

9.3.2 Culture Media

1/4 strength Nutrient Minimal Broth (0.25NB-MM)

Nutrient Broth (Oxoid CM 1)	3.25g
Minimal Media (Difco 0756-17-7)	10.6g
Distilled Water	1000mL

1/4 strength Nutrient Minimal Broth at various a_w (0.20NB-MM/a_w)

Nutrient Broth (Oxoid CM 1)	3.25g
Minimal Media (Difco 0756-17-7)	10.6g
NaCl	% w/w
Distilled Water	approx. 1000mL

Over-strength 0.20NB-MM was prepared in less than 1000mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added by weight. Sterile distilled water was added to make the final volume up to 1000mL.

1/5 strength Nutrient Minimal Broth (0.20NB-MM)

Nutrient Broth (Oxoid CM 1)	2.6g
Minimal Media (Difco 0756-17-7)	10.6g
Distilled Water	1000mL

1/5 strength Nutrient Minimal Broth at various a_w (0.25NB-MM/a_w)

Nutrient Broth (Oxoid CM 1)	2.6g
Minimal Media (Difco 0756-17-7)	10.6g
NaCl	% w/w
Distilled Water	approx. 1000mL

Over-strength 0.25NB-MM was prepared in less than 1000mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl ((determined by reference to Table 3 in Chirife and Resnik, 1984) had been added by weight. Sterile distilled water was added to make the final volume up to 1000mL.

Brain Heart Infusion Agar (BHA)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Technical Agar No.3 (Oxoid L13)	15.0g
Distilled Water	1000mL

After autoclaving, agar medium was cooled to 50°C prior to pouring plates.

Brain Heart Infusion Agar with 0.1% Pyruvate (BHA-P)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Technical Agar No.3 (Oxoid L13)	15.0g
Pyruvic Acid (Sigma P-8574)	1.0g
Distilled Water	1000mL

After autoclaving, agar medium was cooled to 50°C prior to pouring plates.

Brain Heart Infusion Broth (BHI)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Distilled Water	1000mL

Brain Heart Infusion Broth at various a_w (BHI/a_w)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
NaCl	% w/w
Distilled Water	approx. 1000mL

Over-strength BHI was prepared in less than 1000mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added by weight. The medium was autoclaved, then sterile distilled water was then added to make the final volume up to 1000mL.

Brain Heart Infusion Broth with 0.6% added glucose

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Distilled Water	900mL
Glucose (Sigma 7258)	60g
Distilled Water	100mL

Over-strength BHI was prepared in 900mL of distilled water and the medium autoclaved. 60g of glucose was dissolved in 100mL of distilled water. The solution was filter sterilised then added to the overstrength BHI to make the final volume up to 1000mL.

Brain Heart Infusion Broth with 2.0% added glucose

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Distilled Water	600mL
Glucose (Sigma 7258)	200g
Distilled Water	400mL

Over-strength BHI was prepared in 600mL of distilled water and the medium autoclaved. 200g of glucose was dissolved in 400mL of distilled water. The solution was filter sterilised then added to the overstrength BHI to make the final volume up to 1000mL.

Brain Heart Infusion Broth + 8 or 8.5%NaCl with 0.6% added glucose

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
NaCl	% w/w
Distilled Water	approx. 900mL
Glucose (Sigma 7258)	60g
Distilled Water	100mL

Over-strength BHI was prepared in less than 900mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added by weight. The medium was autoclaved. 60g of glucose was dissolved in 100mL of distilled water. The solution was filter sterilised then added to the overstrength BHI to make the final volume up to 1000mL.

Brain Heart Infusion Broth + 8 or 8.5%NaCl with 2.0% added glucose

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
NaCl	% w/w
Distilled Water	approx. 600mL
Glucose (Sigma 7258)	200g
Distilled Water	400mL

Over-strength BHI was prepared in less than 600mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added by weight. The medium was autoclaved. 200g of glucose was dissolved in 400mL of distilled water. The solution was filter sterilised then added to the overstrength BHI to make the final volume up to 1000mL.

Brain Heart Infusion Broth with 85mM lactic acid, pH5.55 (BHI-85)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Lactic acid - 88% (wt/wt) (Univar)	8.71g
Distilled Water	approx. 1000mL

Over-strength BHI was prepared in approximately 800mL distilled water in a volumetric flask, to which lactic acid had been added. The pH was adjusted to 5.55 by addition of 4M NaOH. Media was sterilised by autoclaving at 121°C at 15psi for 15 minutes. After autoclaving, pH was measured and adjusted by addition of sterile 4M NaOH if required. Media was decanted into a sterile 1L volumetric flask and sterile distilled water added to make the final volume to 1000mL.

Brain Heart Infusion Broth + 8%NaCl with 85mM lactic acid, pH5.55 (BHI-85-S)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Lactic acid - 88% (wt/wt) (Univar)	8.71g
Distilled Water	approx. 1000mL

Over-strength BHI plus NaCl was prepared in approximately 800mL distilled water in a volumetric flask, to which lactic acid had been added. The pH was adjusted to 5.55 by addition of 4M NaOH. Media was sterilised by autoclaving at 121°C at 15psi for 15 minutes. After autoclaving, pH was measured and adjusted by addition of sterile 4M NaOH if required. Media was decanted into a sterile 1L volumetric flask and sterile distilled water added to make the final volume to 1000mL.

Brain Heart Infusion Broth with 130mM lactic acid, pH6.1 (BHI-130)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Lactic acid - 88% (wt/wt) (Univar)	13.3g
Distilled Water	approx. 1000mL

Over-strength BHI was prepared in approximately 800mL distilled water in a volumetric flask, to which lactic acid had been added. The pH was adjusted to 6.1 by addition of 4M NaOH. Media was sterilised by autoclaving at 121°C at 15psi for 15 minutes. After autoclaving, pH was measured and adjusted by addition of sterile 4M NaOH if required. Media was decanted into a sterile 1L volumetric flask and sterile distilled water added to make the final volume to 1000mL.

Brain Heart Infusion Broth + 8% NaCl with 130mM lactic acid, pH6.1 (BHI-130-S)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Lactic acid - 88% (wt/wt) (Univar)	13.3g
NaCl	80.0g
Distilled Water	approx. 1000mL

Over-strength BHI plus NaCl was prepared in approximately 800mL distilled water in a volumetric flask, to which lactic acid had been added. The pH was adjusted to 6.1 by addition of 4M NaOH. Media was sterilised by autoclaving at 121°C at 15psi for 15 minutes. After autoclaving, pH was measured and adjusted by addition of sterile 4M NaOH if required. Media was decanted into a sterile 1L volumetric flask and sterile distilled water added to make the final volume to 1000mL.

Columbia Horse Blood Agar (CHBA)

Commercial pre-poured plates (Oxoid PP 2001) were purchased and stored at 4°C.

Eosin Methylene Blue Agar (EMB)

Eosin Methylene Blue Agar-Levine (Oxoid CM69)	37.5g
Distilled Water	1000mL

After autoclaving, the agar medium was cooled to 60 °C and shaken to oxidise the methylene blue and redistribute the precipitate. The agar media was then further cooled to 50°C prior to pouring plates.

Eosin Methylene Blue Agar with 100μg/mL Streptomycin (EMB-S)

Eosin Methylene Blue Agar-Levine (Oxoid CM69)	37.5g
Streptomycin Sulphate stock solution	1mL
Distilled Water	1000mL

After autoclaving, the agar medium was cooled to 60 °C and shaken to oxidise the methylene blue and redistribute the precipitate. The stock solution was added once the medium had cooled to below 50°C, then plates were poured.

MacConkey Agar No. 3 (MAC)

MacConkey Agar No.3 (Oxoid CM 115)	51.5g
Distilled Water	1000mL

After autoclaving, the agar medium was cooled to 50°C prior to pouring plates.

Nutrient Broth (NB)

Nutrient Broth (Oxoid CM 1)	13.0g
Distilled Water	1000mL

Nutrient Broth with 15% v/v glycerol (NB-Gly)

Nutrient Broth (Oxoid CM 1)	13.0g
Glycerol (BDH-Anala R, Prod 10118)	15mL
Distilled Water	1000mL

15mL of glycerol was measured into a volumetric flask then dispensed into a vessel containing the NB powder, but without the distilled water. Due to the viscous nature of the glycerol, small volumes of the distilled water were added to the volumetric flask to "wash out" the glycerol into the vessel containing the NB.

Nutrient Broth at various a_w (NB/a_w)

Nutrient Broth (Oxoid CM 1)	13.0g
NaCl	% w/w
Distilled Water	approx. 1000mL

Over-strength NB was prepared in less than 1000mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added. The medium was autoclaved, then sterile distilled water was then added to make the final volume up to 1000mL.

Nutrient Broth with 100μg.mL^{-1} Streptomycin (NB-1S)

Nutrient Broth (Oxoid CM 1)	13.0g
Streptomycin sulphate stock solution	1 mL
Distilled Water	1000mL

After autoclaving, the medium was cooled to 45°C and the streptomycin sulphate stock solution added.

Nutrient Broth with 200μg.mL^{-1} Streptomycin (NB-2S)

Nutrient Broth (Oxoid CM1)	13.0g
Streptomycin sulphate stock solution	2 mL
Distilled Water	1000mL

After autoclaving, the medium was cooled to 45°C and the streptomycin sulphate stock solution added.

Peptone Water (PW)

Bacteriological Peptone (Oxoid L37)	1.0g
Distilled Water	1000mL

Peptone Water containing 5% NaCl (PWS)

Bacteriological Peptone (Oxoid L37)	1.0g
NaCl	50.0g
Distilled Water	1000mL

Plate Count Agar (PCA)

Plate Count Agar Standard-APHA (Oxoid CM 463)	23.5g
Distilled Water	1000mL

After autoclaving, the agar medium was cooled to 50°C prior to pouring plates.

Plate Count Agar + 5% NaCl (PCA-NaCl)

Plate Count Agar Standard-APHA (Oxoid CM 463)	23.5g
NaCl	50.0g
Distilled Water	1000mL

After autoclaving, the agar medium was cooled to 50°C prior to pouring plates.

Plate Count Agar with 100μg.mL^{-1} Streptomycin (PCA-S)

Plate Count Agar Standard-APHA (Oxoid CM 463)	23.5g
Streptomycin Sulphate stock solution	1mL
Distilled Water	1000mL

After autoclaving, the agar medium was cooled to 50°C and the streptomycin sulphate stock solution added prior to pouring plates.

Physiological Saline (PS)

Sodium chloride	0.85g
Distilled Water	1000mL

Streptomycin sulphate stock solution

Streptomycin sulphate (Sigma S6501)	1.0g
Distilled Water	10mL

The solution was sterilised by passing through a 0.22μ filter. 1mL and 2mL aliquots were stored in sterile McCartney bottles at -20°C. The solution was thawed as required. The solution was not refrozen.

Tryptone Soya Broth + 0.6% Yeast Extract (TSB-YE)

Tryptone Soya Broth (Oxoid CM 129)	30.0g
Yeast Extract (Oxoid L21)	6.0 g
Distilled Water	1000mL

Tryptone Soya Broth + 0.6% Yeast Extract at variable a_w (TSB-YE/a_w)

Tryptone Soya Broth (Oxoid CM 129)	30.0g
Yeast Extract (Oxoid L21)	6.0 g
NaCl	% w/w
Distilled Water	approx. 1000mL

Over-strength TSB-YE was prepared in less than 1000mL of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added. The medium was autoclaved, then sterile distilled water was added to make the final volume up to 1000mL.

9.4 RAW EXPERIMENTAL DATA

A. 9.4.1 Data modelled to produce Eqn. (18)

Mellefont (unpubl.)					Salter (1998)					Presser et al. (1997)									
°C	a_w	pH	[LAC]	GT (h)	°C	a_w	pH	[LAC]	GT (h)	°C	a_w	pH	[LAC]	GT (h)	°C	a_w	pH	[LAC]	GT (h)
25.20	0.999	7.40	0	0.88	7.63	0.997	7.40	0	60.51	20.88	0.998	5.00	100	7.28	22.10	0.994	5.86	0	1.36
25.20	0.995	7.40	0	0.85	10.30	0.997	7.40	0	19.13	20.88	0.997	4.42	25	4.05	22.12	0.989	5.93	200	1.70
25.20	0.991	7.40	0	0.91	12.03	0.997	7.40	0	9.31	20.92	0.998	5.20	100	3.42	22.12	0.992	4.78	50	3.60
25.20	0.988	7.40	0	1.00	13.20	0.997	7.40	0	6.55	20.94	0.991	5.57	100	2.10	22.12	0.994	6.15	0	1.33
25.30	0.984	7.40	0	1.08	14.48	0.997	7.40	0	4.82	20.94	0.991	5.85	100	2.01	22.12	0.994	6.94	0	1.28
25.30	0.980	7.40	0	1.30	15.00	0.966	7.40	0	17.63	20.94	0.991	7.25	100	2.37	22.16	0.991	6.52	100	1.52
25.30	0.976	7.40	0	1.46	15.00	0.969	7.40	0	10.70	20.94	0.998	5.41	100	2.10	22.16	0.991	7.19	100	1.54
25.30	0.972	7.40	0	1.84	15.00	0.972	7.40	0	7.01	20.94	0.998	5.74	100	1.66	22.18	0.992	7.58	50	1.45
25.30	0.969	7.40	0	2.39	15.00	0.976	7.40	0	5.06	20.94	0.997	4.52	25	2.96	22.18	0.994	4.88	0	1.50
25.30	0.965	7.40	0	3.98	15.00	0.979	7.40	0	4.20	20.94	0.997	4.57	25	2.32	22.26	0.989	7.78	200	3.83
25.40	0.961	7.40	0	8.90	15.00	0.982	7.40	0	3.76	20.96	0.998	6.16	100	1.69	22.26	0.992	7.49	50	1.45
25.40	0.998	7.40	0	0.88	15.00	0.985	7.40	0	3.34	20.96	0.997	6.02	25	1.53	22.26	0.994	7.44	0	1.33
25.40	0.995	7.40	0	0.85	15.00	0.991	7.40	0	2.95	20.98	0.989	7.53	200	2.82	22.26	0.994	7.60	0	1.30
25.40	0.991	7.40	0	0.95	15.00	0.994	7.40	0	2.72	20.98	0.989	8.14	200	3.93	22.36	0.991	7.80	100	1.83
25.40	0.987	7.40	0	1.00	16.03	0.997	7.40	0	3.46	20.98	0.997	5.16	25	1.90	22.38	0.991	7.61	100	1.76
25.40	0.984	7.40	0	1.11	17.38	0.997	7.40	0	2.70	21.00	0.997	6.73	25	1.58	22.38	0.992	6.10	50	1.51
25.40	0.980	7.40	0	1.28	18.53	0.997	7.40	0	2.23	21.02	0.991	6.32	100	1.76	22.40	0.989	7.65	200	2.43
25.40	0.976	7.40	0	1.54	20.00	0.971	7.40	0	5.01	21.08	0.997	4.02	0	7.34	22.44	0.992	5.39	50	1.60
25.40	0.973	7.40	0	1.80	20.00	0.971	7.40	0	5.79	21.10	0.989	7.10	200	2.64	22.46	0.989	6.25	200	1.47
25.40	0.969	7.40	0	2.39	20.00	0.974	7.40	0	4.14	21.12	0.991	5.58	100	2.37	22.48	0.994	5.53	0	1.34
25.40	0.965	7.40	0	2.94	20.00	0.974	7.40	0	4.77	21.12	0.997	4.07	0	3.58	22.50	0.991	5.59	100	1.72
25.40	0.962	7.40	0	4.11	20.00	0.977	7.40	0	3.00	21.14	0.989	7.86	200	3.04	22.52	0.992	7.78	50	1.48
25.40	0.958	7.40	0	6.77	20.00	0.977	7.40	0	3.31	21.14	0.997	4.13	0	3.02	22.60	0.989	7.88	200	3.10
25.20	0.997	7.40	0	0.93	20.00	0.980	7.40	0	2.60	21.18	0.997	4.39	0	2.31	22.62	0.994	7.88	0	1.43
25.20	0.994	7.40	0	0.96	20.00	0.983	7.40	0	2.02	21.20	0.997	4.56	50	6.41	22.76	0.991	8.00	100	1.82
25.20	0.990	7.40	0	1.02	20.00	0.983	7.40	0	2.10	21.20	0.997	4.60	0	1.89					
25.20	0.986	7.40	0	1.14	20.00	0.986	7.40	0	1.68	21.22	0.997	4.60	50	5.18					
25.30	0.983	7.40	0	1.37	20.00	0.986	7.40	0	1.88	21.23	0.989	5.77	200	2.91					
25.30	0.979	7.40	0	1.44	20.00	0.988	7.40	0	1.48	21.26	0.997	4.71	50	4.13					
25.30	0.976	7.40	0	1.76	20.00	0.988	7.40	0	1.69	21.26	0.997	5.27	0	1.49					
25.30	0.972	7.40	0	2.16	20.00	0.991	7.40	0	1.54	21.26	0.997	6.88	0	1.44					
25.30	0.969	7.40	0	2.64	20.00	0.991	7.40	0	1.58	21.28	0.986	8.14	500	2.53					
25.30	0.965	7.40	0	3.42	20.00	0.994	7.40	0	1.41	21.30	0.986	7.78	500	2.53					
25.30	0.962	7.40	0	5.31	20.00	0.994	7.40	0	1.47	21.30	0.997	5.08	50	2.02					
25.30	0.958	7.40	0	10.36	20.18	0.997	7.40	0	1.67	21.32	0.989	5.58	200	5.97					
25.10	0.999	7.40	0	0.89	21.50	0.997	7.40	0	1.47	21.32	0.989	6.32	200	2.10					
25.20	0.995	7.40	0	0.88	22.68	0.997	7.40	0	1.18	21.32	0.989	6.72	200	1.59					
25.10	0.991	7.40	0	0.93	24.05	0.997	7.40	0	1.00	21.32	0.989	8.28	200	2.81					
25.20	0.988	7.40	0	1.08	25.00	0.974	7.40	0	1.96	21.38	0.986	6.76	500	7.47					
25.30	0.984	7.40	0	1.13	25.00	0.977	7.40	0	1.56	21.38	0.997	6.13	50	1.41					
25.30	0.980	7.40	0	1.42	25.00	0.980	7.40	0	1.22	21.42	0.989	6.01	200	1.60					
25.20	0.976	7.40	0	1.66	25.00	0.983	7.40	0	1.07	21.56	0.989	7.28	200	2.24					
25.20	0.972	7.40	0	1.99	25.00	0.986	7.40	0	0.95	21.60	0.986	6.86	500	8.13					
25.30	0.969	7.40	0	2.77	25.00	0.988	7.40	0	0.76	21.70	0.989	6.69	200	1.45					
25.30	0.965	7.40	0	4.19	25.00	0.991	7.40	0	0.78	21.70	0.994	5.20	200	6.12					
25.30	0.961	7.40	0	12.65	25.00	0.994	7.40	0	0.78	21.76	0.986	7.75	500	3.09					
25.20	0.998	7.40	0	0.92	25.43	0.997	7.40	0	0.87	21.76	0.994	5.31	200	7.97					
25.20	0.995	7.40	0	0.90	26.70	0.997	7.40	0	0.81	21.82	0.994	5.84	200	1.71					
25.20	0.991	7.40	0	0.95	27.90	0.997	7.40	0	0.65	21.84	0.989	5.65	200	4.32					
25.20	0.987	7.40	0	1.06	29.30	0.997	7.40	0	0.67	21.88	0.986	8.14	500	2.19					
25.20	0.984	7.40	0	1.19	30.00	0.971	7.40	0	1.88	21.94	0.989	6.00	200	1.51					
25.30	0.980	7.40	0	1.40	30.00	0.975	7.40	0	1.63	21.96	0.991	7.51	100	1.84					
25.30	0.976	7.40	0	1.73	30.00	0.978	7.40	0	1.16	21.96	0.992	5.02	50	2.65					
25.20	0.973	7.40	0	2.12	30.00	0.981	7.40	0	0.91	21.98	0.989	7.45	200	2.29					
25.30	0.969	7.40	0	2.55	30.00	0.984	7.40	0	0.92	22.00	0.994	7.14	0	1.29					
25.30	0.965	7.40	0	3.62	30.00	0.987	7.40	0	0.81	22.02	0.992	6.68	50	1.51					
25.30	0.962	7.40	0	5.19	30.00	0.991	7.40	0	0.81	22.02	0.992	7.21	50	1.58					
25.30	0.958	7.40	0	8.16	30.00	0.994	7.40	0	0.77	22.02	0.994	5.14	0	1.59					
25.40	0.951	7.40	0	14.95	30.60	0.997	7.40	0	0.58	22.04	0.991	5.10	100	6.78					
25.20	0.997	7.40	0	0.89	32.08	0.997	7.40	0	0.53	22.04	0.991	7.10	100	1.47					
25.20	0.994	7.40	0	0.92	33.60	0.997	7.40	0	0.50	22.04	0.994	6.58	0	1.36					
25.20	0.990	7.40	0	1.04	34.98	0.997	7.40	0	0.47	22.06	0.989	6.96	200	1.60					
25.30	0.986	7.40	0	1.10	36.70	0.997	7.40	0	0.43	22.06	0.994	4.27	0	2.71					
25.30	0.983	7.40	0	1.27	38.03	0.997	7.40	0	0.42	22.06	0.994	6.14	0	1.36					
25.30	0.979	7.40	0	1.50	40.08	0.997	7.40	0	0.38	22.08	0.989	5.40	200	5.71					
25.00	0.976	7.40	0	1.83	41.85	0.997	7.40	0	0.39	22.08	0.992	5.56	50	1.76					
25.00	0.972	7.40	0	2.45	43.63	0.997	7.40	0	0.38	22.10	0.989	7.28	200	1.96					
25.10	0.969	7.40	0	3.41	45.55	0.997	7.40	0	0.46	22.10	0.991	6.04	100	1.58					
25.00	0.965	7.40	0	7.33	47.43	0.997	7.40	0	1.33	22.10	0.991	6.94	100	1.51					
25.10	0.962	7.40	0	13.06						22.10	0.992	6.01	50	1.45					
25.20	0.958	7.40	0	24.22						22.10	0.992	7.56	50	1.48					

A. 9.4.2. **Growth of *K. oxytoca*, grown in BHI at 25.0 (±0.1)°C, inoculated into pH and lactic acid adjusted BHI broths at various temperatures, determined by turbidimetry.**

Temperature (°C)	pH	Lactic acid (mM)	GT (min)	Lag Time (min)	RLT
0.5	5.55	130	NSG		
5.8	5.55	130	1422.00	1653.00	1.2
9.2	5.55	130	429.40	232.08	0.5
12.3	5.55	130	221.50	174.72	0.8
15.1	5.55	130	150.70	92.74	0.6
17.6	5.55	130	103.70	41.87	0.4
20.4	5.55	130	76.10	33.13	0.4
22.8	5.55	130	59.90	26.86	0.4
25.4	5.55	130	46.60	22.09	0.5
28.0	5.55	130	41.70	18.06	0.4
31.0	5.55	130	32.90	15.04	0.5
33.8	5.55	130	30.30	12.98	0.4
36.7	5.55	130	28.60	13.92	0.5
40.3	5.55	130	30.40	22.37	0.7
44.2	5.55	130	Terminated		
2.9	6.05	85	NSG		
7.2	6.05	85	530.60	510.50	1.0
10.7	6.05	85	238.60	281.54	1.2
13.6	6.05	85	134.50	78.27	0.6
16.4	6.05	85	95.10	12.41	0.1
19.1	6.05	85	66.30	23.34	0.3
21.6	6.05	85	51.30	16.98	0.3
24.3	6.05	85	38.50	12.17	0.3
26.9	6.05	85	33.50	5.00	0.2
29.4	6.05	85	28.80	8.55	0.3
32.1	6.05	85	25.90	19.50	0.8
35.2	6.05	85	21.20	6.17	0.3
38.1	6.05	85	22.20	7.68	0.3
42.0	6.05	85	27.40	5.08	0.2
47.0	6.05	85	4566.00	2326.00	0.5

NSG=Non-sustainable growth

A. 9.4.3 **Isothermal growth (25.4 (±0.4)°C) of *K. oxytoca*, grown in BHI and inoculated into pH and lactic acid adjusted BHI broths at various a_w, determined by turbidimetry.**

a_w	pH	Lactic acid (mM)	GT (min)	Lag Time (min)	RLT
0.988	5.58	130	**58.21**	16.64	0.3
0.986	5.58	130	55.21	17.21	0.3
0.984	5.58	130	57.50	25.37	0.4
0.981	5.58	130	60.76	28.07	0.5
0.979	5.58	130	62.24	35.80	0.6
0.977	5.58	130	65.81	41.37	0.6
0.975	5.58	130	70.50	41.27	0.6
0.972	5.58	130	74.98	64.86	0.9
0.970	5.58	130	84.56	50.23	0.6
0.968	5.58	130	90.38	54.41	0.6
0.966	5.58	130	106.53	69.04	0.6
0.963	5.58	130	114.18	79.82	0.7
0.961	5.58	130	136.96	101.50	0.7
0.959	5.58	130	161.73	118.31	0.7
0.957	5.58	130	194.09	136.93	0.7
0.957	5.58	130	224.79	134.88	0.6
0.952	5.58	130	298.55	153.39	0.5
0.950	5.58	130	346.06	167.06	0.5
0.948	5.58	130	467.33	243.16	0.5
0.943	5.58	130	623.37	173.99	0.3
0.993	6.05	85	47.00	19.21	0.4
0.991	6.05	85	56.20	34.44	0.6
0.988	6.05	85	50.10	21.25	0.4
0.986	6.05	85	53.80	20.15	0.4
0.983	6.05	85	55.30	38.72	0.7
0.981	6.05	85	59.70	47.52	0.8
0.978	6.05	85	63.20	48.08	0.8
0.976	6.05	85	69.70	61.39	0.9
0.973	6.05	85	73.90	67.18	0.9
0.971	6.05	85	86.20	78.77	0.9
0.969	6.05	85	89.80	73.24	0.8
0.966	6.05	85	103.40	84.55	0.8
0.964	6.05	85	122.00	102.93	0.8
0.961	6.05	85	142.80	130.23	0.9
0.959	6.05	85	181.20	119.21	0.6
0.956	6.05	85	205.80	130.73	0.6
0.954	6.05	85	258.30	136.38	0.5
0.951	6.05	85	301.50	163.85	0.5
0.949	6.05	85	451.80	243.73	0.5
0.944	6.05	85	510.90	192.39	0.4

NSG=Non-sustainable growth

Source: Liquid Media	*n*	Temp.	pH	[LAC]	aw	Bias	Acc.	*E.coli* strain	Environment
Barber (1908)	218	10 to 46.8	7.4	0	0.997	0.86	1.17	Bacillus coli	Beef peptone broth
Barber (1908)	24	30.1 to 43.8	7.4	0	0.997	0.83	1.21	Bacillus coli	Beef peptone broth
Barber (1908)	32	30 to 37.5	7.4	0	0.997	0.87	1.15	Bacillus coli	Beef peptone broth
Buchanan & Bagi (1997)	4	12 to 28	5.5 to 7.5	0	0.987	1.55	1.55	O157:H7 933	BHI (adjusted with NaCl,10N HCl or 50%KOH)
Buchanan & Klawitter (1992)	58	10 to 42	4.5 to 8.5	0	0.957 to 0.987	1.38	1.68	O157:H7 cocktail	BHI (adjusted with NaCl,10N HCl or 50%KOH)
Buchanan *et al.*, (1993)	1	12	6.5	0	0.987	1.22	1.22	O157:H7 cocktail	BHI (adjusted with NaCl,10N HCl or 50%KOH)
Doyle & Schoeni (1984)	8	25 to 44.5	7.3	0	0.995	0.43	2.33	O157:H7-932	TSB
Eustace (*pers . comm* . 1998)	5	8 to 37	7.3	0	0.995	0.92	1.16	NCTC 9001-non pathogenic	TSB
Fratamico *et al.*, (1997)	6	37	7	0	0.997	0.82	1.23	O157 cocktail	LB
Gill & Phillips (1985)	11	7.7 to 46	7.4	0	0.993	0.65	1.53	K12 ATCC 23716	BHI
Gill & Phillips (1985)	8	10 to 42.3	7.4	0	0.964	0.81	1.25	K12 ATCC 23716	BHI + 5%NaCl
Glass *et al.*, (1992)	5	37	7.3	0	0.954 to 0.991	0.54	1.85	O157:H7 cocktail	TSB
Glass *et al.*, (1992)	7	37	4.5 to 7.3	0	0.995	0.66	1.52	O157:H7 cocktail	TSB (adjusted with HCl)
Ingraham (1958)	20	8 to 46	7.3	0	0.995	0.62	1.63	K12	TSB
Jason (1983)	2	37	6.5	0	0.996	1.00	1.02	NCIB 9132	Broth'
Jennison (1935)	10	22 to 42	7.4	0	0.997	0.75	1.34	Not stated	NB
Kauppi *et al* ., (1996)	20	8.5 to 12	7.4	0	0.995	1.06	1.21	O157:H7	BHI
Kauppi *et al* ., (1996)	16	8.5 to 12	7.3	0	0.993	0.72	1.52	O157:H7, 0104:H21,O22:H8,0111:NM	TSB
Lowry *et al.*, (1989)	7	8.19 to 40	5.5	130	0.993	1.41	1.41	from chilled sheep livers	Synthetic Meat Medium
Maxcy & Liewen (1989)	5	10 to 30	7.2	0	0.998	0.76	1.32	Not stated	m-Plate Count Broth
Mellefont (*unpubl* .)	46	9.9 to 45.5	7.47	0	0.995	0.89	1.15	SB1-non pathogenic	NB
Palumbo *et al.*, (1995)	3	10 to 37	7.4	0	0.993	0.95	1.23	O157:H7-A9124-1	BHI
Rajkowski & Marmer (1995)	46	8 to 28	5 to 7	0	0.977 to 0.991	0.99	1.46	O157:H7 cocktail	BHI
Salter (1998)	256	7.7 to 47.2	7.4	0	0.997	1.02	1.22	9 STEC strains	NB
Smith (1995)	1	25	7.4	0	0.997	0.89	1.13	SF (sheep feces)	NB
Smith (1995)	1	25	7.4	0	0.997	0.89	1.13	SF (sheep feces)	NB Mg++ (NB + 5x10^3 M MgSO4)
Sutherland *et al.*, (1995)	5	10 to 30	4.49 to 6.97	0	0.954 to 0.991	1.13	1.52	O157:H7 cocktail	TSB
TOTAL	825								

Source: Food	*n*	Temp.	pH	[LAC]	aw	Bias	Acc.	*E.coli* strain	Environment
Buchanan *et al.*, (1993)	1	42	5.9	0	0.98	0.92	1.08	O157:H7 933	Canned tuna
Buchanan *et al.*, (1993)	1	12	6.6	0	0.992	0.52	1.93	O157:H7 933	Canned dogfood
Buchanan *et al.*, (1993)	1	28	6	0	0.976	1.34	1.34	O157:H7 933	Chicken broth
Buchanan *et al.*, (1993)	1	19	6.5	0	0.985	0.80	1.25	O157:H7 933	UHT milk
Gill & DeLacy (1991) **	6	8 to 30	6.5	85	0.997	1.07	1.48	E10 from offal	High pH beef (striploin steaks)
Gill & Newton (1980)	2	20 and 30	5.5	130	0.997	0.76	1.32	Not stated	Meat slices
Gill & Newton (1980)	2	20 and 30	5.5	130	0.997	0.65	1.53	Not stated	Meat slices
Gill & Newton (1980)	9	20 and 30	5.5	130	0.997	0.78	1.43	Not stated	Meat slices+ non-pathogenic psychrotrophs
Gill & Newton (1980) **	4	30	5.5	130	0.997	0.65	1.53	Not stated	Meat slices+ non-pathogenic psychrotrophs
Grau (1983)	10	25	5.6 to 6.91	80	0.99	0.72	1.40	from sheep feces	Beef-thin layers of lean mince
Grau (1983)	1	25	5.6	130	0.997	1.35	1.35	from sheep feces	Fatty tissue
Grau (1983) **	8	25	5.5 to 6.79	80	0.99	0.93	1.33	from sheep feces	Beef-lean pieces
Grau (1983) **	1	25	5.6	130	0.997	1.35	1.35	from sheep feces	Fatty tissue
Kauppi *et al* ., (1996)	14	8.5 to 12	7.2	0	0.986	0.91	1.18	O157:H7, 0104:H21,O22:H8,0111:NM	Autoclaved whole milk
Mellefont (*unpubl* .)	4	10 to 20	6	100	0.997	1.20	1.27	SR M23 (streptomycin resistant)	Sterile raw ground beef
Mellefont (*unpubl* .)	3	10 to 20	6	100	0.997	0.87	1.15	SR M23 (streptomycin resistant)	Low background flora ground beef
Palumbo *et al.*, (1997)	13	8 to 37	7.2	0	0.997	0.54	1.92	O157 cocktail	UHT-pasteurized milk
Palumbo *et al.*, (1997)	4	12 and 15	7.2	0	0.997	0.53	1.90	O157 cocktail	Low background flora pasteurized milk
Palumbo *et al.*, (1997)	3	12	7.2	0	0.997	0.64	1.57	O157 cocktail	High background flora pasteurized milk
Palumbo *et al.*, (1997)	6	12 and 15	5.8	100	0.997	0.60	1.68	O157 cocktail	Irradiated ground beef (Initial 10^3 TVC)
Smith (1985)	1	8.2	6	85	0.997	1.15	1.15	SF	Raw blended mutton
Smith (*pers. comm.*)	67	10 to 40	6	85	0.997	1.10	1.16	SF	Raw blended mutton
Walls & Scott (1997)	6	12 to 35	5.7 to 6.4	85 to 130	0.997	1.05	1.11	O157:H7 cocktail	Raw ground beef (pH adjusted with 3N NaOH)
Wang *et al.*, (1997)	10	8 to 22	6.9 to 7.1	0	0.997	0.65	1.54	O157:H7 cocktail	Unpasteurised milk
TOTAL	178								

**=anaerobic growth conditions applied

A. 9.4.6. Assessment of Eqn. (18) using data for *E. coli* growth presented in Sutherland *et al.* (1995)

	Generation time (hours)						Generation time (hours)				
	Obs.	S	D-m	E 18	PMP		Obs.	S	D-m	E 18	PMP
#1	1.46	0.80	0.90	0.69	0.60	#6	2.50	2.44	3.30	2.16	2.80
TSB	0.58	0.52	0.60	0.45	0.40	BHI	1.20	1.35	1.50	1.65	1.50
	0.49	0.40	0.40	0.30	0.20		1.20	1.64	1.80	1.65	1.20
	0.57	0.40	0.40	0.28	0.20		1.20	1.99	2.20	1.68	1.30
	0.64	0.41	0.40	0.28	0.20		3.40	5.10	5.80	9.65	4.40
	1.08	0.45	0.40	0.32			5.30	3.40	3.70	9.47	2.90
	1.26	0.46	0.40	0.34			4.70	3.80	4.50	9.83	2.50
	1.21	0.47	0.40	0.37			1.10	0.41	0.40	0.48	0.70
#2	0.53	0.49	0.40	0.46			0.40	0.25	0.20	0.36	0.40
NB	0.37	0.45	0.40	0.32			0.30	0.26	0.20	0.36	0.20
	0.35	0.41	0.40	0.28	0.20		0.30	0.46	0.50	0.37	0.20
	0.37	0.40	0.40	0.29	0.20		0.60	0.42	0.40	0.63	0.40
	0.35	0.40	0.40	0.31	0.30		1.40	1.42	1.50	2.33	1.60
	0.47	0.43	0.40	0.35	0.30		11.80	3.44	4.50	3.11	4.20
	0.50	0.46	0.50	0.39	0.30		2.50	1.97	2.10	3.62	2.30
	0.55	0.52	0.60	0.45	0.40		0.60	0.43	0.50	0.91	0.60
	0.77	0.61	0.70	0.53	0.40		0.70	0.54	0.70	1.41	0.80
	0.93	0.73	0.80	0.63	0.50		1.00	0.85	1.30	3.70	1.10
	1.45	0.90	0.10	0.76	0.60		6.20	7.15	6.80	10.41	5.20
	1.60	1.14	1.30	0.94	0.80		0.90	1.10	1.70	3.84	0.90
	2.83	1.50	1.70	1.19	1.10		0.70	0.50	1.00	2.17	0.60
	4.33	2.04	2.20	1.56	1.40		0.70	0.39	0.30	0.45	0.70
	5.83	2.85	3.00	2.13	2.00		0.30	0.27	0.20	0.33	0.20
	6.67	4.12	4.20	3.08	2.70	#7	9.03	11.47	10.00	20.94	
	9.67	6.14	6.10	4.84	3.90	beef	5.35	7.15	6.80	9.09	5.10
	20.00	9.45	8.90	8.71	5.70		5.42	4.61	4.50	5.05	3.50
	41.67	15.00	13.00	20.07			3.70	2.52	2.60	2.65	2.10
#3	1.22	1.22	1.40	0.90	0.80		1.35	1.08	1.20	1.24	1.00
NB	0.77	0.71	0.80	0.55	0.50		0.78	0.36	0.40	0.47	0.40
	0.55	0.50	0.50	0.38	0.30	#8	1.80	1.15	1.30	1.81	1.30
	0.42	0.43	0.40	0.29	0.20	beef	2.10	1.15	1.30	1.81	1.30
	1.08	1.22	1.40	0.90	0.80		1.20	0.36	0.40	0.69	0.50
	0.65	0.71	0.80	0.55	0.50		1.40	0.36	0.40	0.69	0.50
	0.43	0.50	0.50	0.38	0.30	#9	0.30	0.24	0.20	0.30	0.30
	0.33	0.43	0.40	0.29	0.20	mutton	0.37	0.26	0.20	0.36	0.30
	0.43	0.45	0.40	0.27	0.20		0.52	0.34	0.40	0.49	0.40
#4	14.42	8.96	8.40	8.39	5.60		0.78	0.56	0.60	0.75	0.70
broth	2.68	2.89	3.00	2.19	2.10		1.40	1.09	1.20	1.30	1.20
	1.59	1.52	1.70	1.22	1.10		2.60	2.60	2.80	2.77	2.50
	0.68	0.75	0.80	0.66	0.60		6.90	7.57	7.60	9.50	6.10
	0.50	0.49	0.50	0.44	0.40		17.20	11.66	11.00	19.78	
	0.37	0.39	0.40	0.32	0.30	#10	0.72	0.57	0.60	0.97	0.70
	0.33	0.37	0.40	0.29	0.20	beef	1.60	0.65	0.70	0.84	0.50
#5	0.40	0.24	0.40	0.33	0.20		1.53	0.56	0.60	0.84	0.50
TSB	0.40	0.24	0.40	0.37	0.30		1.40	0.55	0.60	0.85	0.60
	0.50	0.26	0.40	0.45	0.40		1.45	0.54	0.60	0.86	0.60
	1.60	0.38	0.80	0.81	0.50		1.20	0.54	0.60	0.93	0.60
	31.70	0.73	1.90	4.13			1.13	0.56	0.60	1.00	0.70
	0.40	0.17	0.30	0.29	0.20		1.10	0.57	0.60	1.14	0.70
	0.40	0.18	0.20	0.29	0.20		1.24	0.58	0.70	1.19	0.70
	0.40	0.10	0.20	0.29	0.30						
	0.50	0.10	0.20	0.30	0.30						

Where #1=Doyle and Schoeni (1984), #2=Ingraham (1958), #3=Jennison (1935), #4=Barber (1908), #5=Glass *et al.*(1992), #6=Buchanan *et al.* (1992), #7=Gill and DeLacy, (1991), #8=Gill and Newton, (1980), #9=Smith (1985), #10=Grau, (1983); TSB=Tryptone Soya Broth, NB=Nutrient Broth, BHI=Brain Heart Infusion Broth, Obs.=Observed, S=Sutherland modified Gompertz model (1995), D=Baranyi D-model, E 18=Eqn. (18) and PMP=Pathogen Modeling Program

A. 9.4.7. Assessment of 3M™ Petrifilm™ *E. coli* Count plates for recovery of *E. coli*

Time(hours)	Viable Count (log cfu.mL^{-1})		
	PCA	EMB	PECC
12°C, a_W 0.960			
0.0	5.61	5.61	5.34
4.0	4.86	4.81	4.48
12.0	5.04	5.04	4.45
16.0	4.97	5.04	4.52
20.0	4.94	4.90	4.49
24.0	5.00	4.98	4.69
28.0	4.94	4.93	4.57
36.0	4.92	4.92	4.54
44.0	4.77	4.74	4.11
52.0	4.75	4.73	4.20
92.0	4.00	3.95	3.70
116.0	3.83	3.78	3.23
236.0	3.11	2.97	2.28
260.0	2.91	2.89	2.52
267.0	2.40	2.40	Not sampled
20°C, a_w 0.980			
277.0	2.49	2.48	Not sampled
281.0	2.28	2.32	Not sampled
283.0	2.26	2.20	Not sampled
285.0	2.26	2.30	1.85
291.0	2.20	2.23	Not sampled
303.0	2.59	2.49	Not sampled
309.0	3.04	3.04	Not sampled
329.0	5.63	5.61	5.28
335.0	5.79	5.81	5.41
338.5	6.20	6.23	5.41
343.5	6.46	6.48	5.78
348.0	8.00	8.04	7.56
350.0	8.70	8.69	8.23
352.0	9.04	9.04	8.76
354.0	9.32	9.32	9.18
363.5	9.79	9.79	9.73
378.0	9.74	9.68	9.58

A. 9.4.8 Growth rate comparison between *E. coli* M23 and *E. coli* SR-M23

Temperature (°C)	Generation time (minutes)	
	E. coli M23	*E. coli* SR-M23
20	305.5	308.1
25	317.8	323.0
30	418.1	443.4
35	542.9	600.3

A. 9.4.9. **The effect of immediate NaCl mediated a_w shifts on *E. coli* SB1 grown isothermally, 25.4 (± 0.4 °C), in NB at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	Lag Time (min)	RLT
0.999 inoculum			
0.999	48.57	4.30	0.1
0.995	44.57	6.90	0.2
0.991	48.76	12.10	0.2
0.988	54.78	21.10	0.4
0.984	56.90	26.60	0.5
0.980	77.97	78.40	1.0
0.976	77.74	110.90	1.4
0.972	104.50	278.80	2.7
0.969	131.90	540.10	4.1
0.965	220.50	1001.50	4.5
0.961	439.20	1534.60	3.5
0.957	Tube Terminated		
0.950	NSG		
0.980 inoculum			
0.998	51.59	29.10	0.6
0.995	42.61	7.58	0.2
0.991	55.48	15.40	0.3
0.987	50.86	-2.42	0.0
0.984	56.43	5.31	0.1
0.980	69.84	8.56	0.1
0.976	80.50	5.93	0.1
0.973	85.75	9.83	0.1
0.969	11.70	2.39	0.0
0.965	143.60	17.74	0.1
0.962	195.50	2.47	0.0
0.958	262.40	-21.32	0.0
0.951	NSG		
0.960 inoculum			
0.997	53.78	104.50	1.9
0.994	52.47	58.96	1.1
0.990	54.20	51.61	0.9
0.986	60.44	40.57	0.7
0.983	81.25	63.39	0.8
0.979	77.54	22.20	0.3
0.976	94.82	33.27	0.3
0.972	118.40	41.80	0.3
0.969	133.50	14.93	0.1
0.965	170.50	19.14	0.1
0.962	251.90	47.92	0.2
0.958	473.40	5.55	0.0
0.951	NSG		

NSG=Non-sustainable growth

A. 9.4.10. The effect of immediate NaCl mediated a_w shifts on 'cell yield' and growth of *E. coli* SB1 at 25.4 (± 0.4 °C), in 0.25NB-MM at various a_w (pH 7.20), determined by turbidimetry.

a_w	GT (min)	Lag Time (min)	RLT	ΔOD
0.997 inoculum				
0.997	49.90	-17.42	0.0	0.812
0.993	62.66	29.29	0.5	0.787
0.990	55.34	23.28	0.4	0.795
0.986	56.30	37.70	0.7	0.783
0.982	69.71	64.85	0.9	0.771
0.979	77.35	59.09	0.8	0.775
0.976	100.99	127.19	1.3	0.720
0.972	143.61	257.23	1.8	0.666
0.968	177.12	497.33	2.8	0.617
0.965	244.85	1007.66	4.1	0.564
0.961	455.82	1869.56	4.1	0.493
0.957	746.88	3007.09	4.0	0.383
0.951	**NSG**			0.185
0.980 inoculum				
0.996	49.37	22.29	0.5	0.821
0.993	63.18	-0.51	0.0	0.824
0.990	62.53	9.91	0.2	0.802
0.986	59.26	0.50	0.0	0.807
0.982	64.51	-1.24	0.0	0.802
0.978	60.45	-5.10	0.0	0.792
0.975	92.23	2.20	0.0	0.737
0.971	91.55	0.94	0.0	0.676
0.967	106.30	-0.66	0.0	0.634
0.965	170.25	3.79	0.0	0.579
0.961	200.29	3.77	0.0	0.520
0.957	231.36	26.77	0.1	0.423
0.950	**NSG**			0.275
0.960 inoculum				
0.996	57.00	96.82	1.7	0.806
0.992	53.92	34.84	0.6	0.816
0.989	66.44	69.61	1.0	0.798
0.985	66.81	30.67	0.5	0.785
0.981	105.14	45.30	0.4	0.773
0.977	104.60	50.11	0.5	0.772
0.975	101.80	-24.40	0.0	0.744
0.971	124.26	57.48	0.5	0.687
0.967	134.79	101.09	0.8	0.627
0.964	161.73	19.87	0.1	0.585
0.960	220.82	9.11	0.0	0.503
0.956	325.61	0.44	0.0	0.412
0.949	**NSG**			0.307

NSG=Non-sustainable growth

A. 9.4.11. **Effect of immediate NaCl mediated a_w shifts on *E. coli* R31 grown isothermally, 25.4 (± 0.4 °C), in NB at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	Lag Time (min)	RLT
0.999 inoculum			
0.999	44.95	-4.36	0.0
0.995	50.07	16.52	0.3
0.991	53.45	15.54	0.3
0.988	67.77	52.56	0.8
0.984	65.52	31.64	0.5
0.980	79.85	60.09	0.8
0.976	93.56	93.25	1.0
0.972	114.70	158.75	1.4
0.969	166.10	329.34	2.0
0.965	228.80	526.09	2.3
0.961	479.90	628.02	1.3
0.957	Tube Terminated		
0.950	NSG		
0.980 inoculum			
0.998	52.37	33.77	0.6
0.995	47.75	7.47	0.2
0.991	47.64	2.57	0.1
0.987	60.52	13.57	0.2
0.984	60.59	-1.49	0.0
0.980	73.14	0.06	0.0
0.976	87.43	0.19	0.0
0.973	122.00	27.23	0.2
0.969	129.60	5.07	0.0
0.965	185.50	8.70	0.0
0.962	224.10	22.39	0.1
0.958	292.30	17.64	0.1
0.951	474.40	-3.69	0.0
0.960 inoculum			
0.997	47.64	57.99	1.2
0.994	49.14	58.88	1.2
0.990	62.66	58.36	0.9
.986	60.73	70.80	1.2
0.983	70.21	73.81	1.1
0.979	82.88	97.87	1.2
0.976	100.00	95.69	1.0
0.972	147.20	134.37	0.9
0.969	178.30	81.01	0.5
0.965	339.20	-0.40	0.0
0.962	457.40	-4.42	0.0
0.958	509.40	-16.14	0.0
0.951	NSG		

NSG=Non-sustainable growth

A. 9.4.12. **Effect of immediate NaCl mediated a_w shifts on *K. oxytoca* grown isothermally, 25.4 (± 0.4 °C), in BHI at various a_w (pH 7.31), determined by turbidimetry.**

a_w 0.996 inoculum				a_w 0.949 inoculum			
a_w	GT (min)	Lag Time (min)	RLT	a_w	GT (min)	Lag Time (min)	RLT
0.996	42.95	13.10	0.3	0.995	45.40	61.28	1.3
0.993	43.79	32.91	0.8	0.992	49.61	66.03	1.3
0.991	44.63	23.30	0.5	0.990	49.96	91.24	1.8
0.988	48.03	29.67	0.6	0.987	55.43	94.98	1.7
0.985	50.80	35.76	0.7	0.984	61.10	106.75	1.7
0.982	59.35	46.65	0.8	0.981	67.12	99.92	1.5
0.980	62.47	65.97	1.1	0.979	70.73	89.60	1.3
0.977	71.75	70.67	1.0	0.976	76.40	113.07	1.5
0.974	79.96	115.09	1.4	0.974	87.58	150.12	1.7
0.971	93.58	209.55	2.2	0.971	102.04	122.80	1.2
0.970	115.46	385.22	3.3	0.969	125.05	142.56	1.1
0.967	130.71	594.71	4.5	0.966	142.46	166.11	1.2
0.964	158.38	813.85	5.1	0.963	172.50	185.82	1.1
0.961	186.97	1070.00	5.7	0.960	210.39	203.12	1.0
0.959	240.53	1388.64	5.8	0.958	265.28	273.23	1.0
0.956	323.87	1638.30	5.1	0.955	348.98	374.63	1.1
0.953	480.99	2190.25	4.6	0.952	531.80	275.00	0.5
0.950	730.03	2765.57	3.8	0.949	928.78	186.00	0.2
0.948	1548.11	4177.06	2.7	0.947	1428.49	282.56	0.2
0.942	NSG			0.941	NSG		

NSG=Non-sustainable growth

A. 9.4.13 **Effect of immediate NaCl mediated a_w shifts on growth of *K. oxytoca* at, 25.4 (± 0.4 °C), in BHI at various a_w (pH 7.20), determined simultaneously by viable count and turbidimetry .**

a_w	GT (min) VC	GT (min) OD	Lag Time (min) Viable count	RLT Viable count
0.992	43.33	40.88	-6.89	0.0
0.984	42.25	55.13	99.16	2.3
0.976	46.25	74.62	300.06	6.5
0.968	54.56	97.80	586.45	10.8
0.960	84.44	130.02	1044.77	12.4
0.953	192.76	227.86	1788.99	9.3
0.949	270.59	346.62	2763.31	10.2
0.945	459.49	737.14	3478.20	7.6

A. 9.4.14. Effect of immediate NaCl mediated a_w downshifts on 'cell yield' and growth of *K. oxytoca* at, 25.4 (± 0.4 °C), in 0.20NB-MM at various a_w (pH 7.20) determined by turbidimetry.

a_w	GT (min)	Lag Time (min)	RLT	ΔOD
0.996	47.61	0.79	0.0	0.921
0.993	51.52	-8.16	0.0	0.952
0.991	48.95	-3.97	0.0	0.961
0.988	52.15	4.80	0.1	0.967
0.986	51.00	15.51	0.3	0.975
0.983	58.74	17.68	0.3	0.965
0.981	63.35	36.46	0.6	0.934
0.978	66.19	39.89	0.6	0.897
0.976	70.92	51.32	0.7	0.883
0.973	85.00	48.72	0.6	0.843
0.971	107.05	103.72	1.0	0.781
0.968	101.26	130.71	1.3	0.739
0.965	149.66	191.59	1.3	0.682
0.963	214.28	299.71	1.4	0.650
0.960	233.41	426.39	1.8	0.602
0.958	278.11	703.44	2.5	0.530
0.955	405.26	931.57	2.3	0.459
0.953	472.88	1136.41	2.4	0.338
0.950	746.58	1441.58	1.9	0.242
0.945	NSG			0.129

NSG=Non-sustainable growth

A. 9.4.15. The effect of immediate NaCl mediated a_w shifts on *S.* Typhimurium grown isothermally, 25.4 (± 0.4 °C), in BHI at various a_w (pH 7.40), determind by turbidimetry.

a_w	GT (min)	Lag Time (min)	RLT
0.993	46.74	24.77	0.53
0.990	53.62	31.98	0.60
0.987	53.69	35.67	0.66
0.984	59.30	82.61	1.40
0.981	60.61	80.74	1.33
0.978	70.46	122.45	1.74
0.975	77.13	161.86	2.10
0.972	Tube Terminated		
0.969	112.20	678.39	6.05
0.966	154.52	955.82	6.19
0.963	206.74	1167.02	5.64
0.960	267.22	1439.50	5.39
0.957	407.95	2001.63	4.91
0.954	611.58	2667.92	4.36
0.951	Growth + clumping		
0.948	Growth + clumping		
0.945	NSG		
0.942	NSG		
0.939	NSG		
0.936	NSG		

NSG=Non-sustainable growth

A. 9.4.16. **The effect of immediate a_w downshifts on *S. aureus* grown isothermally, 25.4 (± 0.4 °C), in NB at various a_w (pH 7.31), determined by turbidimetry.**

a_w	GT (min)	Lag Time (min)	RLT
0.998	60.13	14.13	0.2
0.992	59.53	16.11	0.3
0.986	64.29	21.48	0.3
0.981	65.20	12.70	0.2
0.975	68.96	18.82	0.3
0.969	74.63	25.97	0.3
0.963	78.22	31.43	0.4
0.957	84.17	5.66	0.1
0.952	103.68	23.81	0.2
0.946	116.12	15.64	0.1
0.940	120.73	0.66	0.0
0.934	143.59	-6.71	0.0
0.928	155.41	-2.12	0.0
0.923	207.19	-3.15	0.0
0.917	253.09	-4.89	0.0
0.911	240.09	40.28	0.2
0.905	334.82	46.19	0.1
0.899	468.87	-4.25	0.0
0.894	515.23	85.40	0.2
0.888	640.19	131.05	0.2
0.882	1007.30	-57.71	0.0
0.876	1267.89	375.81	0.3
0.870	NSG		
0.859	NSG		

NSG=Non-sustainable growth

A. 9.4.17 **Effect of immediate NaCl mediated osmotic shifts on *L. monocytogenes* grown isothermally, 25.4 (± 0.4 °C), in TSB-YE at various a_w (pH 7.09), determined by turbidimetry.**

a_w	GT (min)	Lag Time (min)	RLT
0.996	50.38	-6.11	0.0
0.992	61.47	14.29	0.2
0.989	63.29	-0.03	0.0
0.985	73.23	6.54	0.1
0.981	78.98	24.07	0.3
0.977	84.42	39.63	0.5
0.974	92.11	38.68	0.4
0.970	107.65	76.62	0.7
0.966	110.60	57.92	0.5
0.963	157.80	118.93	0.8
0.959	164.21	75.50	0.5
0.955	199.55	95.04	0.5
0.951	259.33	101.02	0.4
0.948	298.75	106.88	0.4
0.944	368.50	203.83	0.6
0.940	415.74	181.87	0.4
0.936	519.10	471.15	0.9
0.933	587.82	601.24	1.0
0.929	858.19	1369.82	1.6
0.922	NSG		

NSG=Non-sustainable growth

A. 9.4.18 **Effect of temperature shift outside the normal physiological range on growth of *E. coli* SB1 in NB, (pH 7.47), for a range of temperatures, determined by turbidimetry.**

44.4 (±0.1°C) inoculum				10.0 (±0.1°C) inoculum			
Temp. (°C)	GT (min)	Lag Time (min)	RLT	Temp. (°C)	GT (min)	Lag Time (min)	RLT
46.6	NSG			47.1	NSG		
44.1	27.5	16.0	0.6	45.5	39.6	-2.1	0.0
42.6	21.4	11.1	0.5	43.9	22.0	3.3	0.2
40.7	18.1	3.4	0.2	41.9	22.5	14.3	0.6
39.2	19.8	8.6	0.4	40.1	22.4	15.9	0.7
37.3	21.2	9.5	0.4	38.8	22.5	10.6	0.5
35.6	23.5	10.7	0.5	37.1	24.4	10.7	0.4
34.1	26.1	15.8	0.6	35.5	25.2	4.2	0.2
32.6	29.0	19.6	0.7	33.6	25.8	5.6	0.2
31.0	29.2	17.4	0.6	32.2	27.4	5.6	0.2
29.7	37.8	22.3	0.6	30.6	31.9	7.4	0.2
27.8	36.3	26.1	0.7	29.1	36.5	10.7	0.3
26.4	38.0	23.3	0.6	27.7	40.4	6.3	0.2
24.9	51.5	47.7	0.9	26.1	47.4	25.5	0.5
23.7	57.6	44.1	0.8	24.5	58.1	33.4	0.6
22.3	69.9	64.5	0.9	23.0	64.1	32.4	0.5
20.7	72.7	42.9	0.6	21.5	75.1	27.9	0.4
19.2	95.7	104.4	1.1	19.9	85.7	57.9	0.7
18.0	110.9	126.8	1.1	18.5	102.1	46.2	0.5
16.8	152.6	204.7	1.3	17.0	132.8	20.8	0.2
15.4	211.5	294.6	1.4	15.3	134.7	5.3	0.0
13.7	278.5	571.7	2.1	13.7	189.5	35.2	0.2
11.5	696.1	1700.1	2.4	12.1	290.6	74.1	0.3
9.9	861.4	2076.0	2.4	10.4	488.7	213.3	0.4

NSG=Non-sustainable growth

A. 9.4.19. Effect of temperature shift from within the normal physiological range, 25.0 (±0.1)°C, on growth of *K. oxytoca* in BHI, (pH 7.28), for a range of temperatures, determined by turbidimetry.

Temperature (°C)	GT (min)	Lag Time (min)	RLT
0.7	NSG		
2.7	NSG		
5.4	1838.00	-213.30	0.0
7.5	586.90	478.23	0.8
9.4	312.10	67.48	0.2
10.8	231.50	205.72	0.9
12.4	160.30	101.86	0.6
14.0	139.50	89.94	0.6
15.2	119.80	86.73	0.7
16.8	92.60	50.32	0.5
17.9	78.00	21.25	0.3
19.3	71.20	26.69	0.4
20.5	67.10	35.10	0.5
21.9	50.50	6.73	0.1
23.1	47.60	14.27	0.3
24.4	39.20	2.39	0.1
25.6	35.90	38.37	1.1
27.5	34.30	3.78	0.1
28.2	29.20	-0.13	0.0
29.5	30.30	9.95	0.3
30.8	30.80	22.26	0.7
32.4	24.50	14.50	0.6
33.8	21.90	4.36	0.2
35.5	20.80	5.97	0.3
37.0	16.90	-1.46	0.0
38.6	18.20	2.46	0.1
40.6	17.70	-1.38	0.0
42.4	24.40	14.97	0.6
44.7	35.10	-0.55	0.0

NSG=Non-sustainable growth

A. 9.4.20. The effect of immediate HCl and NaOH mediated pH shifts on *E. coli* M23 grown isothermally, 25.1 (± 0.1 °C), in NB at various pH, determined by turbidimetry.

pH	GT (min)	LT (min)	RLT	pH	GT (min)	LT (min)	RLT
#1				#3			
3.97	419.6	695.5	1.66	3.23	NSG		
4.04	234.9	296.3	1.26	3.69	NSG		
4.29	175.2	187.7	1.07	4.27	203.3	430.4	2.12
4.43	142.4	101.8	0.71	4.88	91.9	2.0	0.02
4.62	129.1	57.8	0.45	5.14	91.5	-1.4	0.00
4.82	130.9	72.3	0.55	5.53	73.4	-3.5	0.00
5.11	108.3	33.1	0.31	5.86	71.2	-5.8	0.00
5.49	109.0	32.6	0.30	6.15	72.0	-6.0	0.00
6.09	101.5	13.3	0.13	6.14	73.8	2.7	0.04
6.30	94.8	11.4	0.12	6.58	78.7	-5.2	0.00
6.56	92.9	7.8	0.08	6.94	66.2	-5.5	0.00
6.70	94.7	9.77	0.10	7.14	47.9	3.40	0.07
6.85	96.6	9.16	0.09	7.44	76.7	-4.63	0.00
6.99	104.2	15.20	0.15	7.60	68.8	-5.64	0.00
7.1	96.4	9.82	0.10	7.88	85.8	-3.27	0.00
#2				#4			
3.61	NSG			2.94	NSG		
4.10	201.5	231.8	1.15	3.48	NSG		
4.60	103.3	124.4	1.20	3.74	NSG		
4.80	90.3	108.2	1.20	4.02	546.1	503.9	0.92
5.04	75.0	37.0	0.49	4.07	243.1	244.1	1.00
5.17	72.4	31.1	0.43	4.13	214.4	246.6	1.15
5.35	95.6	-7.4	0.00	4.39	146.9	64.5	0.43
5.59	60.3	16.8	0.28	4.60	118.9	41.4	0.35
5.87	59.2	-7.0	0.00	5.27	92.8	19.8	0.21
6.36	70.3	4.2	0.06	6.88	84.7	1.6	0.02
6.79	48.0	3.4	0.07	4.13	220.9	231.1	1.05
7.29	56.9	-7.14	0.00				
7.59	64.2	0.17	0.00				
8.11	64.0	8.05	0.13				

NSG=Non-sustainable growth

A. 9.4.21. **The effect of immediate NaCl mediated a_w shifts on exponential, stationary phase and 'mixed' *S.* Typhimurium grown isothermally, 25.4 (± 0.4 °C), in BHI at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	LT (min)	RLT	a_w	GT (min)	LT (min)	RLT
SP				EXP			
0.995	58.12	17.78	0.31	0.995	53.27	15.94	0.3
0.991	59.62	14.09	0.24	0.991	44.66	12.44	0.3
0.987	72.71	17.08	0.23	0.987	50.25	30.09	0.6
0.984	83.44	24.18	0.29	0.984	60.37	52.80	0.9
0.980	87.59	49.27	0.56	0.980	65.43	79.46	1.2
0.976	103.79	83.98	0.81	0.976	84.85	206.16	2.4
0.972	134.24	156.13	1.16	0.972	130.38	881.68	6.8
0.968	174.37	320.46	1.84	0.968	158.96	1316.10	8.3
0.965	247.38	549.79	2.22	0.965	300.19	2367.07	7.9
0.961	365.30	937.34	2.29	0.961	408.12	3069.27	7.5
0.957	594.11	1128.16	1.90	0.957	TT		
0.949	1220.15	1635.04	1.34	0.949	TT		
90%SP				90%EXP			
0.995	61.51	5.98	0.10	0.995	53.09	8.77	0.2
0.991	63.96	16.13	0.25	0.991	60.55	10.55	0.2
0.987	70.42	20.85	0.30	0.987	61.40	27.18	0.4
0.984	74.61	28.88	0.39	0.984	61.88	41.22	0.7
0.980	86.80	51.88	0.60	0.980	73.27	88.40	1.2
0.976	111.24	100.11	0.90	0.976	90.45	150.53	1.7
0.972	125.39	131.03	1.04	0.972	107.90	316.49	2.9
0.969	171.59	366.86	2.14	0.969	135.28	463.39	3.4
0.965	217.43	529.72	2.44	0.965	228.85	861.16	3.8
0.961	338.54	889.91	2.63	0.961	290.09	1160.95	4.0
0.957	481.50	1185.55	2.46	0.957	422.65	1676.89	4.0
0.950	915.89	1681.72	1.84	0.950	788.97	2549.69	3.2

NSG=Non-sustainable growth TT=Tube Terminated

A. 9.4.22. **The effect of immediate NaCl mediated a_w shifts on exponential (EXP) and stationary phase (SP) *S.* Typhimurium grown isothermally, 25.4 (± 0.4 °C), in BHI at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	LT (min)	RLT	a_w	GT (min)	LT (min)	RLT
EXP				SP			
0.993	56.99	15.98	0.28	0.993	55.82	10.39	0.19
0.990	60.11	32.49	0.54	0.990	60.19	32.40	0.54
0.988	56.62	37.42	0.66	0.988	60.80	35.43	0.58
0.985	61.13	69.84	1.14	0.985	62.37	43.98	0.71
0.982	66.85	82.63	1.24	0.982	69.03	60.29	0.87
0.980	75.91	122.94	1.62	0.980	74.64	77.34	1.04
0.977	74.19	146.84	1.98	0.977	83.21	102.73	1.23
0.974	101.26	218.08	2.15	0.975	102.35	137.61	1.34
0.972	111.10	490.33	4.41	0.972	102.44	181.58	1.77
0.969	126.73	994.02	7.84	0.969	125.59	265.24	2.12
0.967	166.50	1371.61	8.24	0.967	152.59	403.95	2.65
0.964	210.49	1746.34	8.30	0.964	182.56	707.26	3.87
0.961	255.34	2089.50	8.18	0.961	220.61	941.98	4.27
0.959	376.35	2423.98	6.44	0.959	301.07	1216.53	4.04
0.956	582.42	3072.25	5.27	0.956	411.69	1555.04	3.78
0.953	1043.76	3323.87	3.18	0.954	571.49	1971.63	3.45
0.951	1128.52	3522.02	3.12	0.951	837.83	2507.67	2.99
0.948	NSG			0.948	1499.20	3063.85	2.04
0.945	NSG			0.946	NSG		
0.940	NSG			0.940	NSG		

NSG=Non-sustainable growth

A. 9.4.23. **The effect of immediate NaCl mediated a_w shifts on exponential *S.* Typhimurium grown isothermally, 25.4 (± 0.4 °C), in BHI, and BHI with 0.6 or 2.0% added glucose at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	LT (min)	RLT	a_w	GT (min)	LT (min)	RLT
BHI				2.0% glucose #2			
0.994	62.05	40.97	0.7	0.975	85.20	200.67	2.4
0.989	61.62	61.47	1.0	0.974	90.75	356.64	3.9
0.984	61.89	64.59	1.1	0.973	102.02	593.81	5.8
0.979	76.54	106.33	1.4	0.971	18.64	801.59	7.4
0.974	104.32	281.12	2.7	0.970	136.58	1137.61	8.3
0.969	144.58	1128.34	7.8	0.969	TT		
0.965	254.26	2449.64	9.6	0.967	166.04	1629.66	9.7
0.960	433.90	4497.31	9.9	0.966	217.49	2159.21	9.9
0.955	NSG			0.965	TT		
0.945	NSG			0.963	242.45	2685.96	11.1
0.6% glucose				0.962	297.05	3350.97	11.3
0.994	56.72	14.45	0.3	0.961	308.43	3620.46	11.7
0.989	58.89	51.28	0.9	0.959	337.81	4359.48	12.9
0.984	64.71	68.59	1.1	0.958	358.96	4860.73	13.5
0.978	75.72	121.51	1.6	0.957	363.27	5731.48	15.8
0.973	106.35	331.86	3.1	0.955	TT		
0.968	153.70	1420.44	9.2	0.954	492.84	8608.97	17.5
0.963	267.44	2667.49	10.0	0.953	TT		
0.958	464.52	5289.33	11.4	0.951	NSG		
0.952	NSG			0.949	NSG		
0.942	NSG						
2.0% glucose #2							
0.993	58.66	29.63	0.5				
0.988	60.69	67.29	1.1				
0.982	62.48	77.31	1.2				
0.977	77.62	151.03	1.9				
0.972	111.81	544.22	4.9				
0.966	184.40	1630.53	8.8				
0.961	261.60	2979.53	11.4				
0.956	346.49	5938.43	17.1				
0.951	NSG						
0.940	NSG						

NSG=Non-sustainable growth TT=Tube Terminated

A. 9.4.24 **The effect of immediate NaCl mediated a_w shifts on exponential *K. oxytoca* grown isothermally, 25.4 (± 0.4 °C), in BHI with 2.0% added glucose at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	Lag Time (min)	RLT
0.975	72.68	138.46	1.9
0.974	78.70	159.97	2.0
0.973	82.67	234.57	2.8
0.971	94.23	353.26	3.7
0.970	96.23	387.81	4.0
0.969	100.98	487.97	4.8
0.967	110.73	625.94	5.7
0.966	121.46	712.32	5.9
0.965	Tube Terminated		
0.963	152.64	931.69	6.1
0.962	166.24	1049.99	6.3
0.961	185.13	1182.76	6.4
0.959	200.19	1318.13	6.6
0.958	237.64	1511.71	6.4
0.957	255.50	1587.86	6.2
0.955	272.60	1733.42	6.4
0.954	314.82	1735.34	5.5
0.953	371.41	2034.76	5.5
0.951	458.41	2536.31	5.5
0.949	585.35	2903.50	5.0

NSG=Non-sustainable growth

A. 9.4.25. **The effect of immediate NaCl mediated a_w shifts on *E. coli SB1* grown isothermally, 25.4 (± 0.4 °C), in BHI at various a_w (pH 7.40), determined by turbidimetry.**

a_w	GT (min)	Lag Time (min)	RLT
0.993	45.45	4.49	0.10
0.991	46.18	17.88	0.39
0.986	54.63	30.08	0.55
0.986	52.73	34.39	0.65
0.984	60.50	55.72	0.92
0.982	59.43	57.38	0.97
0.980	67.82	75.31	1.11
0.977	74.25	104.73	1.41
0.975	88.81	107.05	1.21
0.973	91.96	150.64	1.64
0.970	114.35	211.24	1.85
0.968	126.91	306.66	2.42
0.966	161.33	450.77	2.79
0.964	202.74	727.87	3.59
0.961	213.85	961.75	4.50
0.960	362.01	1636.00	4.52
0.957	Growth + clumping		
0.955	Growth + clumping		
0.952	NSG		
0.948	NSG		

NSG=Non-sustainable growth